Determining genetic diversity and regulation of sexual compatibility in *Colletotrichum lentis* Damm, the causal agent of anthracnose on *Lens culinaris* (Medik.)

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ABSTRACT

Anthracnose of lentil caused by the fungal pathogen *Colletotrichum lentis* is an economically important disease in Western Canada. The pathogen population is divided into two races (0 and 1) and two sexual incompatibility groups (IG-1 and IG-2). Resistance to anthracnose race 1 is found in cultivated *Lens* cultivars whereas for the more aggressive race 0, higher levels of resistance have been reported only from wild *Lens* species. Furthermore, *C. lentis* seems to only possess one (*MAT1-2*) of the two mating type idiomorphs commonly present in heterothallic ascomycete fungi with the typical bipolar mating system. The purpose of this study was to verify the phylogenetic relationship between race 0 and 1 isolates of *C*. *lentis* and to sequence and characterize the *MAT1-2* of *C. lentis*. A morphological, multi-locus phylogenetic and host-range study was conducted with isolates of *C*. *lentis*, *C. truncatum* (from various host species and the epitype)*, C. destructivum, C. dematium*, *C. higginsianum, C. linicola* and *C*. *lindemuthianum*. Sequence data from six conserved loci displayed 100% identity for *C. lentis* isolates of both races that formed a single cluster separate from other *Colletotrichum* species including *C. destructivum,* the epitype of *C. truncatum* and isolates from other hosts identified as *C. truncatum*. Conidia of *C. lentis* were slightly falcate with obtuse apices compared to cylindrical conidia with rounded ends of *C. destructivum,* and longer lunate to falcate conidia of the epitype *C. truncatum.* Host range tests undertaken on *Lens culinaris*, *Pisum sativum*, *Cicer arietinum*, *Vicia faba*, *Glycine max*, *Phaseolus vulgaris*, *Phaseolus lunatus*, *Trifolium pratense*, *Medicago sativa*, *Medicago truncatula*, *Brassica chinensis* and *Arabidopsis thaliana* under controlled environmental conditions revealed that the host ranges of *C. linicola* and *C.*

higginsianum overlapped with that of lentil isolates. In contrast, the epitype specimen of *C. truncatum* was pathogenic on *Pisum sativum*, *Phaseolus vulgaris*, *T. pratense* and *Medicago sativa,* but not on *L. culinaris*. All *Colletotrichum* spp. infected *Medicago truncatula* and all but the lentil isolates caused disease on *G. max*. The mating type gene *MAT1-2* of *C. lentis* contained two introns and three exons and an open reading frame of 726 bp coding for a putative protein of 241 amino acids including the high mobility group (HMG) domain characteristic of the *MAT1-2* in fungi. The *MAT1-2* nucleotide sequences of *C. lentis* isolates were identical irrespective of IG. An isolate from each of the two IGs, CT-21 (IG-2), CT-30 (IG-1) and a co-culture of CT-21 and CT-30 was used to study the expression levels of *MAT1-2* at seven different *in vitro* time points (0h, 6h, 12h, 18h, 24h, 36, 48h after inoculation in glucose yeast media) and investigate for possible alternative splicing events. *MAT1-2* expression for CT-21, CT-30 and the co-culture was observed at all seven time points indicating that it is constitutively expressed, and no differences in the transcript size were seen, ruling out the possibility of a splicing event.

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LIST OF ABBREVIATIONS

AAFC	Agriculture and Agri-Food Canada
ACT	Actin
β-TUB	Beta tubulin
CDC	Crop Development Centre
CHS-1	Chitin synthase 1
EF1α	Elongation factor 1 alpha
FAO	Food and Agriculture Organization of the United Nations
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GYM	Glucose yeast medium
HIS3	Histone 3
HMG	High mobility group
IG	Incompatibility group
ITS	Internal transcribed spacer
OMA	Oatmeal agar medium
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
RAPD	Random Amplified Polymorphic DNA
RT-PCR	Reverse Transcriptase PCR

CHAPTER 1

INTRODUCTION

Lentil (*Lens culinaris* Medik.) is a dicotyledonous, self-pollinating, diploid (2n=14) pulse crop grown predominantly in North America, India, Pakistan, Bangladesh, Egypt, Greece and Italy. Lentil cultivation in Canada began in 1969 and in a span of nearly four decades the country has become the largest producer and exporter of lentil in the world (FAO, 2010). Lentil is subjected to various fungal diseases around the world and in Canada, anthracnose, ascochyta blight, sclerotinia white mould and botrytis stem and pod rot and stemphylium blight are the major fungal diseases that can result in significant crop losses (Morall, 1997; Podder et al. 2013). Among them anthracnose of lentil is currently a major concern on the Canadian prairies (Vail and Vandenberg, 2011). Although anthracnose on lentil has been reported from other parts of the world such as Bulgaria (Kaiser et al.1998), USA (Venette et al. 1994), Pakistan (Morrall, 1997), Syria (Bellar and Kebabeh, 1983) and Brazil (Baldanzi et al.1988), for reasons unknown, this disease appears to have developed into an economically important problem only in Canada.

Anthracnose of lentil in Western Canada was identified for the first time in Manitoba in 1987 and was reported in Saskatchewan in 1990 (Morall, 1991). The fungal pathogen responsible was ambiguously identified from two different sources as *Colletotrichum truncatum* (Morall, 1988) and *C. destructivum* (Platford, 1988) causing confusion about its identity. A host of previous studies employing molecular and morphological approaches involving the comparison of Canadian lentil isolates with isolates of *C.*

truncatum from other plant species and with other closely related *Colletotrichum* spp. have been undertaken. These studies have indicated that the lentil isolates belong to the destructivum clade rather than the truncatum clade (Damm et al. 2009; O'Connell et al. 2012), are closely related to *C.destructivum* and *C.higginsianum* (Liu et al. 2007; Forseille et al. 2011) and are a distinct group separable from *C. truncatum* isolates from other host plants (Ford et al. 2004; Gossen et al. 2008). A recent description of the entire *Colletotrichum destructivum* species complex using multilocus phylogeny resulted in the renaming of *C. truncatum ex* lentil as *C. lentis* (Damm et al. 2014).

Colletotrichum is regarded as a genus of asexual species and the anamorph is the prevalent form found in nature with rare observations of the teleomorph (Vaillancourt et al. 2000). *Colletotrichum lentis* is no exception since its teleomorph has not been found in the field to date (Armstrong-Cho and Banniza, 2006). However, based on disease resistance screening (Buchwaldt et al. 2004) and *in vitro* mating studies (Menat et al. 2012) the *C. lentis* population from Western Canada has been divided into two pathogenic races (0 and 1) and two sexual incompatibility groups (IG-1 and IG-2). Race 1 isolates are less virulent than race 0 isolates and sources of resistance against race 1 isolates have been identified in cultivated lentil (Buchwaldt et al. 2004). In contrast, high levels of resistance for race 0 isolates were primarily found in wild lentil species (Tullu et al. 2006) as compared to *L. culinaris* accessions (Shaikh et al. 2013). Intriguingly, all field isolates tested to date that belong to IG-1 are exclusively race 0 isolates, whereas IG-2 comprises isolates of both race 0 and race 1 (Menat et al. 2012). These observations suggest that race 0 isolates may be a distinct population that co-evolved

with wild lentil species, which developed resistance to this population, and that race 0 isolates may have switched or expanded host range to cultivated lentil species.

Classical mating studies suggests that *C. lentis* is a heterogenically compatible species following a bipolar mating system (Armstrong-Cho and Banniza, 2006) and the presence of the two incompatibility groups IG-1 and IG-2 strengthens the hypothesis (Menat et al. 2012). However, amplification of the high mobility group (HMG) domain encoded by the *MAT1-2* idiomorph of the mating type (MAT) locus, and attempts to amplify the conserved α1-box encoded by the *MAT1-1* idiomorph, revealed the presence of the HMG box in isolates of both incompatibility groups whereas the α1-box was not detected. This contradicts the typical heterothallic bipolar mating system and suggests that *C. lentis* has an unusual mating system (Menat et al. 2012).

Based on the linkage between race and incompatibility group identity among field isolates, and resistance to the races in different species of *Lens*, it was hypothesized that Western Canadian *C. lentis* isolates from race 0 and race 1 represent two distinct taxa. It was also hypothesized that the mating system of *C. lentis* is governed through complementary mutations in *MAT1-2* as proposed in the model of 'unbalanced heterothallism' (Wheeler, 1954). The specific research objectives of this research study were:

1. (a) to examine the relationship between race 0 and race 1 isolates of *C. lentis* from Western Canada using six molecular loci, (b) to investigate the relationship of *C. truncatum* ex. *Matricaria perforata* with the *C. lentis* isolates and other

Colletotrichum species and (c) to study the host range of isolates of *Colletotrichum lentis*, *C. truncatum*, *C. dematium*, *C. destructivum*, *C. higginsianum*, *C. linicola*, *C. lindemuthianum* under controlled environmental conditions.

2. (a) to sequence and characterize *MAT1-2* of *C. lentis* isolates from both incompatibility groups (IG-1 and IG-2), (b) to study the expression profile of *MAT1-2* in *C. lentis* and (c) to investigate the possibility of alternative splicing in the *MAT1-2* gene of *C. lentis*

CHAPTER 2

REVIEW OF LITERATURE

2.1. *Colletotrichum*

2.1.1. The genus *Colletotrichum*

Colletotrichum is a genus of the class Sordariomycetes that belongs to the Ascomycota division of the fungal kingdom. Species in the genus *Colletotrichum* are known to cause blight or anthracnose on a large number of field crops (Bailey and Jeger, 1992). The name *Colletotrichum* was first used by Corda in 1831 to reference the species *C. lineola* found on a plant belonging to the Apiaceae in the Czech Republic. This species was considered to be synonymous with *C. dematium* but was recently shown to be a separate species (Damm et al. 2009). Past accounts suggested *Vermicularia* to be an earlier name for *Colletotrichum* and records of *Colletotrichum* species have also been grouped in the genus *Gloeosporium* (reviewed by Canon et al. 2012). In the seminal works of von Arx (1957, reviewed by Canon et al. 2012) a little over 700 species were shown to be a part of the *Colletotrichum* genus, which was a significant number considering that it was first described in 1831. This could be attributed to the fact that *Colletotrichum* spp. were considered to be host-specific plant pathogens, hence a report of symptoms on a new host would result in the description of a new species. By strictly taking into account only morphological and cultural characteristics, von Arx managed to significantly reduce the species count from over 700 to 11, and even those were still regarded as species aggregates rather than individual species.

Colletotrichum spp. have developed a range of life styles. The majority are plant pathogens, but there are also a few which are human pathogens (Sutton, 1992; TeBeest et al. 1997; Cano et al. 2004; Kumar and Hyde, 2004; Photita et al. 2004), as well as, endophytes and saprophytes (Damm et al. 2009; Prihastuti et al. 2009). *Colletotrichum* spp. are responsible for causing a wide range of diseases in plants from all over the world resulting in substantial crop damage and yield loss (Sutton,1992; Hyde et al. 2009), and making it one of the most extensively studied fungal genera along with *Fusarium, Phytopthora* and *Rhizoctonia* (Latunde-Dada, 2001).

The same species can sometimes have more than one lifestyle, as seen with *Colletotrichum fructicola* and *Colletotrichum siamense*, which behave as pathogens, endophytes and epiphytes on coffee berries and numerous other host plants (Prihastuti et al. 2009). *Colletotrichum* spp. can infect a plant at any stage affecting mostly aerial plant parts and stems. In addition to anthracnose, fungi of this genus are also responsible for causing other diseases, such as sugarcane root rot, berry disease of coffee and brown blotch of cowpea. *Colletotrichum* can also cause up to 100% post-harvest losses in fruits resulting from rots during storage (Prusky, 1996). In general, *Colletotrichum* is perceived as an asexual genus with rare documentation of the sexual morph in nature (Vaillancourt et al. 2000). Members displaying the sexual morph were classified in the genus *Glomerella*, but after the recent acceptance of the one fungus - one name concept for fungi displaying pleomorphism in the International Code of Nomenclature for Algae, Fungi and Plants in Melbourne (2013), *Colletotrichum* is the name to be used henceforth (Hawksworth et al. 2013).

2.1.2. Infection process of *Colletotrichum* pathogens

There are two main sources of inoculum, conidia present in the acervuli and ascospres present in the ascus (for species that are sexually reproducing under field conditions). Both, conidia and ascospores are enclosed in a hydrophilic matrix often called the spore matrix (Nicholson and Moraes, 1980). Ascospores are either released forcibly from the ascus or passively due to breakdown of the ascus cell wall, and both conidia and ascospores are dispersed either through water splash or by wind (Bailey et al. 1992).

The deposition of spores on the plant surface is the initial step toward disease (Hamer et al. 1988). Conidia or ascospores produced by *Colletotrichum* spp. adhere to the plant surface (Young and Kauss,1984) although the mechanism is not very well understood as there is no evidence of adhesives being produced by *Colletotrichum* spp. as seen in the case of *Magnaporthe grisea* (Hamer et al. 1988). Appressorium formation, which is an essential step for infection, takes place immediately after spore deposition and germination on the plant surface (Parberry, 1981). Usually the appressoria are attached directly to the conidia, but may sometimes be attached to a germ-tube or be present at hyphal tips (Bailey et al. 1992). Appressoria are a morphologically distinguishing character in the genus *Colletotrichum* (Sutton, 1968). Melanin is present in the appressoria walls of *Colletotrichum* spp. as a result of protein synthesis (Suzuki et al. 1981) and it imparts a black color to the appressoria, which is essential for penetration of plant tissue (Kubo et al. 1982). Cone shaped structures called germ-pores are often seen in the appressorial wall of some species during penetration, which are thought to exert pressure and facilitate penetration (Wolkow et al. 1983). In contrast to spores, the

adhesion mechanism of appressoria on the plant surface is well studied (Bailey et al. 1992). Appressorial adhesion helps the pathogen to not only stay in contact with the host, but also places the infection hyphae at the penetration site.

The act of penetrating a plant surface by a fungal pathogen is accomplished either by directly penetrating the leaf cuticle or by entering through a natural opening or wound. Direct penetration is more commonly seen in *Colletotrichum*, but entry through wounds has also been reported in a few cases where wound formation is necessary for infection (Agrios, 1988). A latent period after successful penetration seems to exist for *Colletotrichum* spp., and is characteristic for many storage diseases of fruits in the tropical regions. Fungal growth seems to be initially restricted to the epidermis, but latency is no longer seen as the fruits ripen which may be due to formation of new tissues that enhance fungal development (Prusky and Plumbey, 1992). Penetration is considered to be a very important step in the infection cycle which occurs after formation of an appressorium. Successful penetration of the cuticle in the absence of an appressorium has also been demonstrated (Brown, 1975). Advocates of cuticular penetration by *Colletotrichum* spp. have put forth three mechanisms, one involving the use of mechanical force (Mercer et al. 1971; Katoh et al. 1988), another involving secretion of cell wall degrading enzymes by the pathogen (Dickman and Patil, 1986) and a third involving a combination of mechanical force and cell wall degrading enzymes (Bailey et al. 1992).

The rate of initial infection and colonization by a pathogen is directly related to its pathogencity. Many *Colletotrichum* species display a hemibiotrophic mode of infection

which involves an initial symptomless or biotrophic phase followed by a destructive or necrotrophic phase, and are thus called intracellular hemibiotrophs (O'Connell et al. 1985; Politis and Wheeler, 1973). Some *Colletotrichum* spp. referred to as subcuticular intramural pathogens do not invade the cells after penetrating the cuticle, but hyphae grow intercellularly underneath the cuticle (Luttrell, 1974). The intracellular hemibiotrophic mode of infection in its symptomless biotrophic phase involves the formation of a primary hyphal structure which is either restricted to a single epidermal cell or which spreads to neighbouring epidermal cells. This is followed by the destructive or necrotrophic phase in which the primary hyphae give rise to narrow secondary hyphae that then invade the neighbouring cells and tissues, eventually killing them and causing water soaked lesions (Latunde-Dada and Lucas, 2007).

2.1.3. Taxonomic uncertainty in the *Colletotrichum* genus

The genus *Colletotrichum* has been the subject of extensive morphological and molecular taxonomic studies worldwide due to its highly uncertain and confusing taxonomy. It is considered to be made up of nine main clades namely *acutatum, boninense, dematium, destructivum, graminicola, gloeosporioides, orbiculare, spaethianum, and truncatum* (Canon et al. 2012). The identification of a new species based on the host from which the isolates originated irrespective of whether it is morphologically distinguishable from other species has contributed to the ambiguity of species delimitation in the genus *Colletotrichum*.

Traditionally, classification of species by taxonomists was done on the basis of morphology, taking into consideration features such as the shape and size of conidia

and appressoria, setae, sclerotia, the presence or absence of the telomorphic state, and colony characteristics like color, texture and growth rate when grown on artificial medium (Simmonds,1965; Sutton,1992). However, the low number of distinguishing characters in the genus *Colletotrichum* has placed limitations on resolving species, for example, the lack of a sexual stage in the life cycle of most species (Hyde et al. 2009). Variation in colony morphology among species due to environmental factors rendered morphological analysis inadequate. As a consequence, molecular techniques are being used in support of morphological studies to better identify taxa within *Colletotrichum* (Sreenivasaprasad et al. 1996; Abang et al. 2002).

Colletotrichum dematium was first isolated in France from a thistle in the Apiaceae family and was also found on plants of the Solanaceae family (Damm et al. 2009). It has been recorded as a pathogen of numerous hosts like *Capsicum annuum* (Than et al. 2008), *Solanum lycopersicum* (Bello, 2000), soybean (Fakir, 1979) and *Fagus crenata* (Sahashi et al. 1995). Anthracnose symptoms caused by this species have also been reported on amaryllids by Yang et al. (2009). Descriptions on the width of conidia of *Colletotrichum dematium* isolates differ significantly, making this characteristic unhelpful for characterization of the pathogen. Although no type specimen was deposited when the species was originally described, an epitype was designated recently which permits comparison with newly collected isolates (Damm et al. 2009).

Colletotrichum destructivum was reported by O'Gara in 1915 from red clover in the Fabaceae family. In his work, Von Arx (1957, reviewed in Canon et al. 2012) declared *C. destructivum* to be an individual species, but this claim was subsequently refuted

(Sutton, 1980) and the species was thought to be synonymous with *Glomerella cingulata*. *Colletotrichum destructivum* and *C. lindemuthianum* were thought to be synonymous because they shared similar morphological characteristics related to appresorial size and conidial length. However they were shown to be separate species on the basis of dissimilar culture characteristics, conidial width and different teleomorphs (Manadhar et al. 1986). Based on morphology, sequence data for the rDNA-ITS region and mode of infection on *Arabidopsis thaliana*, O'Connell et al. (2004) considered *C. higginsianum* to be a synonym of *C. destructivum*. *Colletotrichum destructivum* has also been shown to be part of the species complex comprising *C. lincola* based on sequence similarities of 97-99% for the domain 2 (D2) and ITS-2 regions of rDNA, similar modes of infection and morphological features that support this classification (Latunde-Dada and Lucas, 2007).

Although more sequence data has become available, issues with accurate species assignment or identification through multigene analysis still arise primarily because of misrepresented sequence data deposited in Genbank (Crouch et al. 2009; Cai et al. 2009) along with the lack of type or epitype specimens (Hyde et al. 2009). Epitypes have been designated for 42 *Colletotrichum* species since the process of epitypification began in 2007 (Hyde et al. 2009). Traditional morphological analysis in combination with advanced molecular techniques are considered the best approach for understanding the genus *Colletotrichum* and its species (Cannon et al. 2000; Cai et al. 2009), and using these approaches, the genus is now being revised (Damm et al. 2009).

2.1.4. Molecular phylogenetic analysis

The concept of species has been divided into either theoretical or operational species concepts by Mayden (1997). The operational species concepts are a more useful tool for the identification of a species because they follow a set of rules or standards which could include morphology, mating or genetic makeup, whereas in the theoretical species concepts no such guidelines are given. Particularly in fungi it has been seen that operational species concepts, such as the phylogenetic species concept based on variation in the nucleotide sequences of individuals, is a suitable tool for evolutionary studies. The phylogenetic species concept can be applied in both sexual and asexual fungi. The use of more than one polymorphic locus in species identification reduces the possibility of misclassifying individuals into a greater number of species (Taylor et al. 2000). A very critical step in phylogenetic studies is selection of the region to be amplified and sequenced, as selecting a highly conserved or variable region is undesirable (Swofford and Olsen, 1990).

Ribosomal DNA (rDNA) is often used in phylogenetic studies because the presence of conserved sequences within the genes simplifies the task of designing primers used for the polymerase chain reaction (Kocher et al. 1989; Hillis et al. 1990). Each subunit of a ribosome consists of two components, rRNA and protein. In eukaryotic organisms the 40s subunit is made up of a single rRNA (18s) and 30 proteins whereas the 60s subunit has three rRNA (5s, 5.8s and 28s) and 40 proteins. The rDNA unit consists of rRNA genes and two internal transcribed spacers, ITS-1 and ITS-2, and a single external

transcribed spacer, ETS, at the 5'-end of the rDNA. A non- transcribed spacer, NTS, is present between the two rDNA units. The 18s, 5.8s and 28s rRNA genes are present on a single transcriptional unit whereas the 5s rRNA gene is situated at a different location in the genome. The NTS has a positive influence on the rate of transcription and the ITS helps in formation of a functional rRNA (reviewed by Hills and Dixon, 1991). Owing to their variable nature, the spacer regions (ITS-1 and 2) are used extensively for identification of species or strains (Saghai-Maroof et al. 1984). Sequence comparison studies of the ITS-2 spacer region have also been used to correctly identify different species of *Colletotrichum (*Sherriff et al. 1995). In contrast to the ITS-2 region, the ITS-1 region seems to have higher sequence variability in isolates belonging to the same or different species of *Colletotrichum* (Sreenivasaprasad et al. 1996).

The first study in *Colletotrichum* involving molecular analysis of more than one locus was carried out in *C. acutatum*, using β tubulin (TUB2) and histone 4 (HIS4) in addition to the traditional ITS locus (Talhinhas et al. 2002). The high mobility group (HMG box) protein encoded by the mating type locus MAT1-2 in the Ascomycota has also been amplified and sequenced in diversity studies at the subgeneric level of closely related clades, revealing high interspecific and low intraspecific variation (Du et al. 2005).

The glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) gene of eukaryotes is involved in glycolysis and has also been employed to study phylogeny in certain fungal pathogens. The use of non-coding regions of a gene is useful in estimating variation among isolates of the same species as these regions possess remarkable variation within a species (Carbon et al. 1999). A 200 bp intron region of the *GAPDH* gene was

used for the first time in a phylogenetic analysis in *C*. *acutatum* using isolates from the USA and New Zealand (Guerber et al. 2003).

Other loci like chitin synthase 1 (CHS-1), histone3 (His3) and a partial actin sequence (ACT) have been used in characterization of *Colletotrichum* spp. with curved conidial structures responsible for anthracnose of herbaceous hosts (Damm et al. 2009). A combination of elongation factor alpha 1 (EF1α) and actin (ACT) gene sequences resulted in higher phylogenetic resolution than using the ITS alone, distinguished isolates of *C. panacicola* of Korean ginseng as a separate taxon from otherwise morphologically similar *Colletotrichum* spp. (Choi et al. 2011). The number of loci presently used to identify *Colletotrichum* spp. is on the rise, maybe because the traditional ITS regions are too evolutionarily conserved to differentiate between closely related taxa.

2.1.5. The *Colletotrichum* genome

The genome sequences of four *Colletotrichum* species have been generated to date: *C*. *fructicola* (Nara-gc5), *C. graminicola* (M1.001), *C. higginsianum* (IMI349063) and *C*. *orbiculare* (104-T) (O'Connell et al. 2012; Gan et al. 2013). *Colletotrichum graminicola* was the only species sequenced using Sanger dideoxy technology whereas Roche 454 and Illumina methods were employed to sequence the other three species. The genome assembly size of *C. orbiculare* (88.3 Mb) is larger in comparison to *C*. *fructicola* (55.6 Mb), *C. graminicola* (57.4 Mb) and *C. higginsianum* (53.4 Mb), and all four *Colletotrichum* genomes are larger than the average genome size (38 Mb) of the Pezizomycotina. The large size of the *C. orbiculare* genome is attributed to the

presence of AT-rich sequences that make up almost 50% of the genome assembly, distributed amidst the coding regions (Gan et al. 2013). The number of predicted genes ranges from 12,006 - 16,172, which are distributed across 10 major chromosomes in all four *Colletotrichum* species. *Colletotrichum fructicola* (14,993) possesses almost twice the number of multi-copy genes as *C. graminicola* (7,475), indicating that perhaps the genome of *C. graminicola* has gone through fewer events of gene duplication than the rest. Surprisingly, a very low level of conserved synteny exists among the four genomes with *C. fructicola* and *C. orbiculare* sharing 40% synteny, and *C. graminicola* and *C. higginsianum* only 35% (O'Connell et al. 2012; Gan et al. 2013).

2.1.6. *Colletotrichum lentis*

Among the four most severe diseases that affect the lentil crop, anthracnose caused by *C. lentis* has the highest economic impact. It was shown to cause significant crop damage and yield losses in Canada, especially in the provinces of Saskatchewan and Manitoba (Buchwaldt et al. 1996; Anderson et al. 2000). *Colletotrichum lentis* can also infect faba bean, vetch, grass pea and field pea causing varying levels of disease severity (Buchwaldt et al. 2004). The typical symptoms associated with *C. lentis* are early development of lesions on young leaves and stems with the appearance of severe brown coloured lesions at flowering causing leaf drop and stem wilting. The fungus has the ability to survive as microsclerotia in crop debris for periods as long as 4 years (Buchwaldt, 1996).

The existence of two pathogenic races of *C. lentis* in Western Canada was reported based on seven different accessions of lentil (Buchwaldt et al. 2004). It was

hypothesized that race 0 is the more aggressive race due to the lack of avirulence genes, whereas race 1 is less aggressive due to presence of at least one avirulence gene. Microscopy studies by Armstrong-Cho et al (2012) revealed no qualitative difference in the infection process between isolates of the two races. The varieties of lentil grown in Canada exhibit partial resistance toward race 1 isolates so farmers employ strategies such as fungicides and crop rotation to control the disease (Chongo and Bernier 1999).

The species appears to prefer an asexual mode of reproduction on lentil as the telomorph for this species has not been reported from the field. The *in vitro* induced sexual stage of *C. lentis* was reported by Armstrong-Cho and Banniza (2006). *Colletotrichum lentis* does not appear to be very diverse. Studies comparing isolates of lentil from Canada using 18-25S rDNA showed only a 0.2% sequence dissimilarity of the ribosomal DNA across all lentil isolates (Ford et al. 2004). The two pathogenic races of *Colletotrichum lentis* can be easily distinguished based on noticeable differences in disease severity on lentil (Buchwaldt et al. 2004), but sequence analysis of the rDNA-ITS region failed to distinguish between them (Forseille et al. 2011).

Colletotrichum lentis displays a hemibiotrophic mode of infection. Conidia of *C. lentis* on lentil germinated within 12 hours post inoculation (hpi) with a higher germination rate observed for the more aggressive race 0 isolates compared to race 1 isolates (Armstrong-Cho et al. 2012). Penetration of the cuticle was observed within 24 hpi and primary hyphae that developed inside the cell were sac-like and possessed many lobes confined to a single epidermal cell for around 48 hpi. Secondary hyphal structures were

observed growing from the multilobed primary hyphae to invade neighbouring cells and tissues, which resulted in the development of chlorotic water soaked anthracnose lesions on inoculated leaves. Aggregation of primary hyphae was observed 72 hpi and monosetate acervuli containing abundant conidia were seen 96 hpi. (Chongo et al. 2002)

2.2. Mating in filamentous ascomycetes

2.2.1. Mating systems in fungi

The fungal division Ascomycota contains organisms that produce their progeny in a sac-like structure called the ascus. Ascomycota have the ability to reproduce sexually as well as asexually. The type of reproduction is influenced by factors like nutrition, temperature and light. A large number of filamentous Ascomycota tend to remain in the haploid form throughout their lifecycle producing only conidia. During sexual reproduction special spore-bearing structures of different shapes develop that are referred to as fruiting bodies and contain asci which upon opening can discharge sexual ascospores over large distances (Nelson, 1996).

The nuclei of filamentous Ascomycota do not fuse immediately after plasmogamy, but remain separate in one cytoplasm and undergo division several times before karyogamy of nuclei of the opposite mating type occurs, unlike acomycetous yeasts in which the nuclei fuse immediately after plasmogamy. These observations arise from mating studies carried out in *Neurospora crassa, Saccharomyces cerevisiae* and *S. pombe*, as their sexual stage can be easily replicated and manipulated under laboratory conditions and because of the availability of mutants (Perkins et al. 1982; Lenon et al. 1992).

Traditionally, based on the structural organization of the fruiting structure, genera of the Ascomycota were broadly divided into six groups, namely Hemiascomycetes, Laboulbeniomycetes, Pyrenomycetes, Loculoascomycetes, Plectomycetes and Discomycetes. Sequence data of the 18s rDNA of species in these groups supported this traditional classification (Berbee and Taylor, 1992). However the Ascomycota classification has been updated and it is shown to be divided into three main subphyla, Taphrinomycotina which consists of Neolectomycetes, Pneumocystidomycetes, Schizosaccharomycetes and Taphrinomycetes; Saccharomycotina which consists of Saccharomycetes, and Pezizomycotina which consists of Arthoniomycetes, Dothideomycetes, Eurotiomycetes, Laboulbeniomycetes, Lecanoromycetes, Leotiomycetes, Lichinomycetes, Orbiliomycetes, Pezizomycetes and Sordariomycetes (Lumbsch and Huhndorf, 2007). Fungi in the Ascomycota display homothallic, heterothallic or pseudohomothallic types of mating. Homothallism refers to self-fertilizing species and heterothallism to cross-fertile ones. The phenomenon of mating type switching has been reported in the Ascomycota, particularly in *S. cerevisiae* and *S. pombe* (Herskowitz et al. 1992). Rodriquez and Owen (1992) reported a unidirectional mating type switch in a few filamentous fungi of the Pyrenomycetes and Discomycetes and also showed that the heterothallic fungus *Glomerella musae* could have more than two mating types.

2.2.2. The mating type (MAT) locus

The mating type genes regulate the process of fertilization and reproduction that take place between fungi of distinct mating types (Kronstad and Staben, 1997). Heterothallic Ascomycota are known to follow a bipolar mating system which involves the presence of two alleles at a single locus called the MAT locus. The two alleles of the mating type gene are called idiomorphs because they have dissimilar DNA sequences that produce different protein products (Metzenberg and Glass, 1990). Classically, the mating type genes are referred to as *MAT1-1* and *MAT1-2*. The protein products encoded by the mating type genes have conserved nucleic acid binding regions which are considered to have a regulatory effect on transcription post-fertilization (Herskowitz,1989). The HMG box of the MTa-1 in *N. crassa* (corresponding to MAT1-2) was shown to bind to DNA sequences (Philley and Staben, 1994).

Models have been developed to describe the regulation of proteins present in the nuclear membranes of fusing nuclei by mating products (Zickler et al. 1995). Dimerization of protein products of opposite mating types is considered a mechanism to recognize self from non-self in Ascomycota and Basidiomycota (Herskowitz, 1989; Metzenberg, 1990). Mating type mutants of *P. anserina* were able to fertilize, but exhibited an inability to fuse with nuclei of the opposite mating type, often resulting in fusion with other mutated nuclei that gave rise to uniparental progeny as opposed to diparental ones seen in wild type mating (Zickler et al. 1995).

The idiomorphic nature of the MAT locus is a common feature of fungal mating type genes. The presence of dissimilar sequences at the MAT locus is responsible for

specifying the mating type of an individual. The MAT locus can carry a single or multiple genes which would differ between opposite mating types (Metzenberg and Glass, 1990). *Neurospora crassa* was the first filamentous ascomycete to have its MAT region sequenced. It revealed the presence of three genes: *mt* A-1, *mt* A-2 and *mt* A-3 within the 5.3 kb MAT locus of mt A idiomorphs, whereas the 3.2 kb MAT locus of mt a idiomorphs contained a single gene, *mt* a-1 (Glass et al. 1988; Ferreira et al. 1996). Mutational studies have revealed that *mt* A-1 and *mt* a-1 are required for mating (Saupe et al. 1996), whereas *mt* A-2 and *mt* A-3 may have a role post mating (Glass and Lee, 1992). The mat^+ and mat^- idiomorphs of *P. anserina* show homology to mt a and mt A of *N. crassa* in terms of gene number and function. The mat^+ idiomorph contains a single gene, *FPR*1, and the mat^- idiomorph consists of three genes, *FMR*1, *SMR*1 and *SMR*2.

2.2.3. Pheromones and pheromone receptors

Pheromones and pheromone receptors are integral to the regulation of mating in fungi as they are responsible for inducing the mating response between partners. *Saccharomyces cerevisiae* has served as a model organism to unravel the process of pheromone signaling in ascomycetes (Jones and Bennett, 2011). The a and α factors are two specific pheromones produced by MAT a and MAT α individuals, respectively, that bind to membrane bound G-protein coupled receptors of the opposite mating type cell (Nakayama et al. 1985). The a factor is a typical fungal pheromone whereas the α factor is exclusive to the ascomycetes (Singh et al.1983). The genes for the two pheromones and their receptors are present in individuals of both mating types,

although they are not linked to the MAT locus, as seen in basidiomycetes, and their expression is solely dependent on mating type (Martin et al. 2011).

It has been demonstrated for heterothallic species that pheromone production is vital for male fertility, but not required for female fertility. Gene expression studies in *P. anserina* revealed that pheromone genes were only expressed in microconidia. The male requires pheromones to attract the female, whereas the female only needs to have the pheromone receptors for fertilization (Coppin et al. 2005; Kim and Borkovich, 2006). In contrast, homothallic species such as *Sordaria macrospora* where genes for both the pheromones and receptors are expressed in an individual; probably as both mating type genes are present in each nucleus, a low level of self- fertility is achieved even after knocking out both pheromone genes while allowing expression of the two receptor genes (Mayrhofer et al. 2006). In *Fusarium graminearum*, individuals without the pheromone and pheromone receptor genes were seen to possess self- fertility suggesting that a pheromone-receptor interaction may not be necessary to initiate signaling in this mating pathway (Lee et al. 2008).

2.3. Prologue to Chapter 3

The following research chapter describes a study to determine the relationship between isolates of *Colletotrichum lentis* from two pathogenic races and sexual incompatibility groups and with other closely related *Colletotrichum* spp. by employing four different approaches that are generally used for species identification in the genus *Colletotrichum*. A multi-locus phylogeny using six conserved loci was performed along with examining the conidial morphology of isolates of *C. lentis* and closely related *Colletotrichum* spp. Host range testing and classical mating experiments were also conducted to evaluate the ability of *Colletotrichum* spp. isolates to infect various leguminous and brassicaceous plant species and assess the isolates based on the biological species concept, respectively.

CHAPTER 3

GENETIC DIVERSITY AND HOST RANGE OF COLLETOTRICHUM LENTIS CAUSING ANTHRACNOSE OF LENTIL IN WESTERN CANADA

3.1. Introduction

The emergence of plant diseases due to human activities such as the domestication of crops and the movement of crops through global commerce has become a major concern worldwide as it provides opportunities for pathogens to move along with those crops, or for indigenous pathogens to expand their host range to these new plant species. Around 30% of these diseases are estimated to be caused by plant pathogenic fungi, and their emergence or spread has generated massive interest in identifying and delimiting the fungal species responsible for the outbreak of novel plant diseases (Giraud et al. 2010). A disease is categorized as emergent if there is evidence of recent increase in pathogen virulence on a new host from a locale not previously reported (Gladieux et al. 2011). Host-range expansion or host-shifts are the strategies generally used by indigenous plant pathogens to infect new hosts, leading to the development of new diseases on those species. In the former scenario, the pathogen retains the ability to infect both the original and the new host, whereas in the latter scenario the pathogen only retains the ability to infect its new host. It has been proposed that pathogen populations that have the potential to infect both agricultural and non-agricultural host plants acquire virulence as a result of migration to the cultivated plant population (Burdon and Thrall, 2008).

An estimated 1. 5 million species are believed to be part of the fungal kingdom (Hawksworth, 1991) and the concepts used to define a species have evolved over time from the morphological and biological to the ecological and phylogenetic species concepts. Each species concept employs certain criteria: the morphological species concept focuses on differences in morphology, the biological species concept utilizes the phenomenon of reproductive isolation, the ecological species concept is based on adaptation to a certain environment or host and the phylogenetic species concept takes into account variation in nucleotide sequences (Taylor et al. 2000). The biological species concept that states 'species are a group of natural populations that can interbreed' is the most widely used concept to define a species (Mayr, 1963), but has limitations in the kingdom Fungi where asexual or homothallic species cannot be delimited on this basis (Taylor et al. 2000). Furthermore, some fungal species display interspecific fertility, for example in the genus *Ascochyta*, where sexually compatible crosses were obtained between isolates of *A. fabae* and *A. lentis* (Kaiser et al. 1997).

Lentil was introduced to Canada relatively recently with the first cultivation dating back to 1969 (Morall, 1997). The first published report of anthracnose on lentil in Western Canada is from 1986-87 in Manitoba, and the causal agent was identified as *Colletotrichum truncatum* which was known to cause anthracnose on soybean (Morrall 1988). Its identification as *C. truncatum* has been questionable since its detection, as an isolate from lentil had already been sent to the Commonwealth Mycological Laboratory, U.K., in 1983 to be identified as *C. destructivum* (Platford 1988), but lack of live type specimens at that time prevented clarification. Molecular and morphological comparison

of Canadian lentil isolates with isolates of *C. truncatum* from other plant species and with other *Colletotrichum* spp. revealed that the lentil isolates could be separated from all other non-lentil isolates and *Colletotrichum* spp. based on the shape of conidia, 18-25S rDNA sequences and RAPD markers (Ford et al. 2004; Forseille et al. 2011). Interestingly, isolates recovered from *Matricaria perforata*, tentatively identified as *C. truncatum*, were shown to cause disease symptoms on *Lens culinaris* (Peng et al. 2005) and were inseparable from *C. lentis* isolates based on conidial dimensions, but were shown to be distinct based on a phylogenetic analysis using ITS sequences (Forseille et al. 2011). Sequence data of the ITS1 region indicated that lentil isolates were closely related to *C.destructivum* and *C.higginsianum* (Liu et al. 2007; Forseille et al. 2011) and were even considered to be a specialized form of *C. destructivum* based on the similarities in the ITS and D2 sequences, infection strategies and morphological features (Latunde-Dada and Lucas 2007). A study on host specificity and latent infection of Canadian lentil isolates and *C. truncatum* isolates from soybean and *Matricaria perforata* also supported separation of the anthracnose pathogens from different hosts into distinct species (Gossen et al. 2009). Comparison of multi-locus sequence data of a lentil isolate with the recently described epitype of *C. truncatum* confirmed that lentil isolates from Canada belong to the *C*. *destructivum* species complex rather than to *C. truncatum* (O'Connell et al. 2012). As a consequence, the lentil isolates were recently re-classified into a new species named *C. lentis* (Damm et al. 2014).

The origin of *C. lentis* is unknown, and it has been hypothesized that it was either introduced on lentil seed from another country, and / or that it jumped host from a native legume species to lentil (Morrall 1997). Two races were differentiated among 50 isolates of *C. lentis* from Western Canada when screening for anthracnose resistance in lentil (Buchwaldt, 2004). Resistance to isolates of the less virulent race 1 was identified in the cultivated lentil species and hypothesized to be triggered by avirulence genes in that race, whereas race 0 isolates were virulent on all accessions (Buchwaldt, 2004). The greatest level of resistance to race 0 isolates was identified initially in wild species of *Lens*, within accessions of *L. ervoides* (Brign.) from the tertiary gene pool (Tullu et al. 2006), although sources of resistance in *L. culinaris* were reported recently (Shaikh et al. 2013). Classical *in vitro* mating studies divided the *C. lentis* isolates into two incompatibility groups (IG-1 and IG-2) with IG-1 comprising exclusively race 0 isolates whereas IG-2 contained both race 0 and 1 isolates (Menat et al. 2012). The existence of two pathogenic races (Buchwaldt et al. 2004) and two incompatibility groups (Menat et al. 2012) in the relatively young *C. lentis* suggests a repeated introduction and/or host-switch.

The objectives of this study were (1) to utilize six loci for examining the phylogenetic relationship between race 0 and race 1 isolates of *C. lentis* from Western Canada, (2) to investigate the relationship of *C. truncatum* ex. *Matricaria perforata* with the *C. lentis* isolates and other *Colletotrichum* species based on this extended number of loci, and (3) to study the host range of isolates from *Colletotrichum lentis*, *C. truncatum*, *C.*

dematium, *C. destructivum*, *C. higginsianum*, *C. linicola*, *C. lindemuthianum* under controlled environmental conditions.

3.2. Materials and Methods

3.2.1. Isolates

A total of 33 isolates of *Colletotrichum* spp. were included in this study (Table 3.1). Among those, 20 had been designated as *C. truncatum* in previous studies, 19 of which were from *L. culinaris* (14 field isolates from Saskatchewan and Manitoba, 5 ascospore-derived isolates) and 1 from *Matricaria perforata*. *Lens culinaris* isolates belonging to two different races (races 0 and 1) (Buchwaldt et al. 2004; Armstrong-Cho et al. 2012) and incompatibility groups (IG-1 and IG-2) (Menat et al. 2012) were selected. Canadian isolates from *Glycine max* and *Matricaria perforata*, and a *C. linicola* isolate provided by Agriculture and Agri-Food Canada (AAFC), Saskatoon Research Centre were included in this study. The epitype specimen of *C. dematium*, the epitype specimen and a second isolate of *C. truncatum* were obtained from the Centraalbureau voor Schimmelcultures (CBS), Fungal Biodiversity Centre, Utrecht, The Netherlands. The isolates of *C. destructivum* were obtained from the National Mycological Herbarium of Canada, Ottawa (DAOM), *C. lindemuthianum* from AAFC Harrow, and *C. higginsianum* from CABI Europe UK Centre, Egham, UK.

Table 3.1. *Colletotrichum* spp. selected for multilocus sequencing and assessment of conidial morphology

Isolate	Host Plant	Species	Race ID	IG [d]
CT-21	*Lens culinaris*	*Colletotrichum lentis*	1	IG-2
CT-28	*Lens culinaris*	*Colletotrichum lentis*	1	IG-2
CT-30	*Lens culinaris*	*Colletotrichum lentis*	0	IG-1
CT-31	*Lens culinaris*	*Colletotrichum lentis*	0	IG-2
CT-32	*Lens culinaris*	*Colletotrichum lentis*	0	IG-1
CT-34	*Lens culinaris*	*Colletotrichum lentis*	0	Infertile
CT-35	*Lens culinaris*	*Colletotrichum lentis*	1	IG-2
CT-37	*Lens culinaris*	*Colletotrichum lentis*	0	IG-2
CT-39	*Lens culinaris*	*Colletotrichum lentis*	1	IG-2
CT-43	*Lens culinaris*	*Colletotrichum lentis*	1	IG-2
CT-44	*Lens culinaris*	*Colletotrichum lentis*	0	IG-1
CT-58	*Lens culinaris*	*Colletotrichum lentis*	1	IG-2
CT-60	*Lens culinaris*	*Colletotrichum lentis*	0	IG-2
CT-233	*Lens culinaris*	*Colletotrichum lentis*	0	IG-2
GT-14	*Lens culinaris*	*Colletotrichum lentis*	0	IG-1
GT-60	*Lens culinaris*	*Colletotrichum lentis*	1	IG-1
GT-95	*Lens culinaris*	*Colletotrichum lentis*	0	IG-1

Table 3.1. (Continued). *Colletotrichum* spp. selected for multilocus sequencing and assessment of conidial morphology

Isolate	Host Plant	Species	Race ID	IG [d]
GT-150	*Lens culinaris*	*Colletotrichum lentis*	1	IG-1
GT-155	*Lens culinaris*	*Colletotrichum lentis*	1	IG-2
CT-49	*Glycine max*	*Colletotrichum truncatum*	-	-
CT-53	*Glycine max*	*Colletotrichum truncatum*	-	-
CT-56	*Glycine max*	*Colletotrichum truncatum*	-	-
CT-64	*Matricaria perforata*	*Colletotrichum truncatum*	-	-
CBS151.35 [a]	*Phaseolus lunatus*	*Colletotrichum truncatum*	-	-
CBS119189	*Phaseolus lunatus*	*Colletotrichum truncatum*	-	-
CBS125.25 [b]	*Eryngium campestre*	*Colletotrichum dematium*	-	-
IMI349063	*Brassica chinensis*	*Colletotrichum higginsianum*	-	-
DAOM225582	*Trifolium pratense*	*Colletotrichum destructivum*	-	-
DAOM225583	*Trifolium pratense*	*Colletotrichum destructivum*	-	-
DAOM225584	*Trifolium pratense*	*Colletotrichum destructivum*	-	-
DAOM225585	*Trifolium pratense*	*Colletotrichum destructivum*	-	-
CLA01	*Convolvulus arvensis*	*Colletotrichum lincola*	-	-
CLO2 [c]	*Phaseolus vulgaris*	*Colletotrichum lindemuthianum*	-	-

a- Epitype specimen of the *C. truncatum*

b- Epitype specimen of the *C. dematium*

c- The outgroup for phylogenetic analysis

d- Incompatibility group

3.2.2. Phylogenetic analysis of multi-locus sequence data

Isolates were cultured on oatmeal-agar medium (OMA: 30 g oatmeal flour [Quick Oats Robin Hood, Markham, ON], 8.8 g granulated agar, 1 L distilled water) and incubated at 22°C for 10-14 days. To obtain mycelia for extraction of DNA, a small amount of fungal mycelium of each isolate was transferred from the OMA plates into flasks of 50 mL yeast glucose medium (1 g $NH_4H_2PO_4$, 0.2 g KCl, 0.2 g $MgSO_4,7H_2O$, 10 g glucose, 5 g yeast extract, 0.01 g $ZnSO_4,7H_2O$, 0.005 g $CuSO_4,5H_2O$, 1 L distilled water). The liquid cultures were incubated on a shaker for 2-3 days at 21-23°C and 130 rpm. Mycelia of isolates were harvested by filtration, stored for 2 days at -80°C, and lyophilized for 1 day (Labconco FreeZone 7753022, Labconco Corp, Kansas City, MO). The lyophilized mycelium of each isolate was pulverized by adding two sterile glass beads to each 2 mL microcentrifuge tube and using a bead shaker for 10 min at 10 VDC (Leeson, Mississauga, ON). DNA was extracted with the DNeasy plant mini kit for DNA extraction (Qiagen Inc, Mississauga, ON) following the manufacturer's protocol.

The DNA samples were diluted with sterile distilled water to a final concentration of 20 ng μl^{-1} for PCR. Six loci were targeted for amplification: 5.8s ribosomal gene and its flanking regions ITS1 and ITS2, partial sequences of actin (ACT), β tubulin (β-TUB), histone 3 (his3) and chitin synthase 1 (CHS-1), and the 200 bp intron of the glyceraldehydes-3-phosphate dehydrogenase gene (GAPDH). For this purpose forward and reverse primers, V9G+ITS4 (5.8S/ITS1/ITS2) (Hoog et al. 1998; White et al. 1990), BT2Fd+BT4R/T1+Bt-2b (β-TUB) (Woudenberg et al. 2009; O'Donnell and Cigelnik 1997; Glass and Donaldson 1995), CYLH3F+CYLH3R (CHS-1) (Crous et al. 2004),

CHS-79F+CHS-354R (CHS-1) (Carbone and Kohn 1999), GDFI+GDRI (GAPDH) (Guerber et al. 2003), ACT-512F+ACT-783R (Carbone and Kohn 1999) were used. The PCR amplifications were done using a Bio-Rad S1000^{TM} thermal cycler (Bio-Rad Laboratories Inc. California, USA), with a final reaction volume of 50 µl. The reaction mix for ACT, β-Tub, CHS-1, GAPDH and HIS3 was made up of 2.5 µl of 10 ng genomic DNA, 1x PCR buffer (Invitrogen, Carlsbad, CA), 2 mM $MgCl_2$, 20 µM of dNTP (Invitrogen, Carlsbad, CA), 0.2 µM of forward and reverse primer, 0.5% DMSO and 1 U of Taq DNA Polymerase (Invitrogen, Carlsbad, CA). PCR reaction conditions consisted of an initial denaturation step of 5 min at 94°C was followed by 40 cycles at 94°C for 30 s, 52°C for 30 s and 72°C for 30 s, and a final elongation step at 72°C for 7 min. The ITS reaction consisted of 2 µl of 10 ng diluted genomic DNA, 1x PCR buffer (Invitrogen, Carlsbad, CA), 1 mM $MgCl_2$, 40 µM of dNTP, 0.2 µM of forward and reverse primer, 0.5% DMSO, and 0.5 U of Taq DNA Polymerase (Invitrogen, Carlsbad, CA). PCR reaction conditions consisted of an initial denaturation step at 94°C for 5 min was followed by 35 cycles at 94°C for 30 s, 48°C for 30 s and 72°C for 1 min, and a final elongation step at 72°C for 7 min. The PCR products were separated on a 1.5% agarose gel in 1x Tris-acetate-EDTA (TAE) buffer (pH 8.0). Gel Red (Biotium, Inc, CA) was used to stain and visualize the DNA bands under UV light and the size of the PCR products was estimated using the 1Kb Plus DNA Ladder (Invitrogen, Carlsbad, CA). All PCR products were gel extracted using an EZ-10 Spin Column DNA Gel Extraction Kit (Bio Basic Canada Inc, Markham, ON) following the manufacturer's protocol and stored at -20°C until sequencing at the National Research Council (NRC), Saskatoon, SK, Canada.

Sequence data of each of the six loci as well as the combined data were used for phylogenetic analysis. DNA sequences of each fungal isolate were used to perform a multiple sequence alignment in MEGA 5.2 (Molecular Evolutionary Genetic Analysis) (Tamura et al. 2011), using the CLUSTALW program to assemble and further manually edit the sequence data. Maximum likelihood and Bayesian inference approaches were used to create phylogenetic trees using MEGA 5.2 and MrBayes v. 3.2.2 (Ronquist and Huelsenbeck 2003), respectively. The selection of best-fit nucleotide substitution models using the Bayesian information criterion (BIC) for each dataset was carried out in PartitionFinder V1.1.1 (Lanfear 2012). The preliminary analyses of individual datasets in PartitionFinder V1.1.1 (Lanfear 2012) suggested that the genes were congruent, permitting the nucleotide sequence alignments of all the six genes to be concatenated using Mesquite V 2.75 (Maddison and Maddison 2011).

The maximum likelihood analysis was executed in MEGA 5.2 on each of the individual genes, as well as the concatenated dataset with the nucleotide substitution models chosen based on the models selected in PartitionFinder V1.1.1 for each of the seven datasets. The strength of the phylogenetic trees generated was tested by 1000 bootstrap replications. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join (NJ) and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach, and then selecting the topology with superior log likelihood value. A discrete gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I]). The tree

topologies of the individual genes were similar to the topology of the combined six-gene tree (data not shown). The Bayesian Interference analysis was performed to determine Bayesian posterior probabilities with a Markov Chain Monte Carlo algorithm and the nucleotide substitution models determined using PartitionFinder V1.1.1 for each gene region. The analysis of four Markov Chain Monte Carlo chains was run twice for 5 x 10^7 generations with posterior sampling done for every 1000 generations. The initial 25% generations were regarded as burn-in phase on the basis of the standard deviation of split frequencies and the performance scale reduction factors, hence were discarded and the remaining were used to determine posterior probabilities in the majority rule consensus tree.

3.2.3. Conidial morphology

All isolates were cultured on OMA medium and incubated at 22°C for 5-7 days. Conidia were viewed with a Zeiss Axioskop 40 microscope (Zeiss Canada, Toronto, ON, Canada) using a x40 objective lens. A total of 40 measurements for conidial length and width were determined using AxioVision 4.9.1 (Carl Zeiss Vision Imaging Systems)

3.2.4. Host range study

Host range studies were conducted with the following 12 plant species: *L. culinaris (*lentil cvs. Eston, CDC Robin), *Pisum sativum* (field pea cv. CDC Amarillo), *Cicer arietinum* (chickpea cv. CDC Luna), *Vicia faba* (faba bean cv. Fatima), *Glycine max* (soybean cv. AC Harmony), *Phaseolus vulgaris* (common bean cv. CDC Jet), *Phaseolus lunatus* (lima bean),*Trifolium pratense* (red clover cv. Altaswede), *Medicago sativa* (alfalfa cv. AC Grazeland), *Medicago truncatula* (barrel medick), *Brassica rapa*

subspecies *Chinensis* (Chinese cabbage) and *Arabidopsis thaliana* (thale cress ecotype Columbia). Five to eight seeds for each plant species were sown in 95 x 95 mm plastic pots filled with a soil-less mixture (Terra-Lite Redi-Earth®, Scotts-Sierra Horticultural Products Co., Marysville, Ohio, USA). Two weeks after planting, plants were thinned to 4 per pot. The plants were maintained in a growth chamber (Model PGV 56, Conviron, Winnipeg, MB) at 22/16°C day/night with a photoperiod of 16 h light and 8 h dark. Fertilization was done two weeks after seeding and then once a week with 20-20-20 NPK + micronutrients at a concentration of 3 g L^{-1} water.

Nine isolates (CT-21, CT-30, CT-53, CBS151.35, CBS125.25, IMI349063, DAOM225584, CLAO1 and CLO2) were cultured on OMA and grown at 22°C in an incubator (Sanyo Versatile Environmental Test Chamber Model MLR-350H, Sanyo Electric Co., Ltd., Gunma, Japan) for 7-10 days, followed by harvesting the conidia from individual culture plates with deionized water to prepare conidial suspensions at a final concentration of 1 x 10^5 mL^{-1}. Plants of each species except *B. chinensis* were inoculated with all nine isolates 4 - 6 weeks post-seeding. All pots were sleeved with plastic wraps in order to promote high humidity during incubation and to avoid cross-contamination. An airbrush sprayer was used to spray the plants with approximately 2 mL of conidial suspension per plant. All plants were incubated in a mist chamber at approximately 90 - 100% RH for 48 h, after which the pots were covered with transparent plastic bags for 3 days. *Arabidopsis thaliana* and *B. chinensis* were inoculated with isolate IMI349063 separately with an extended incubation time of 96 h to promote disease development. Disease severity ratings of leaves and stems were taken

10 days post-inoculation using a quantitative rating scale ranging from 0 (0%) to 10 (91 to 100% disease severity) with 10% increments. A total of four replications over time were carried out for each isolate-host combination, and the entire experiment was conducted twice. Severity ratings on stems and leaves were highly correlated except for *A. thaliana* and *B. chinensis* that did not show stem lesions, so only leaf data is presented. Surface sterilized diseased leaf and stem tissues were plated on potato-dextrose agar (Difco^{TM}) and incubated at room temperature to fulfil Koch's postulate. Data were transformed into percentages using mid-class values and were analyzed using the mixed model procedure in SAS (Version 9. 3, SAS Institute Inc., Cary, NC). Isolates, host plants and their interaction were considered fixed factors, and repeats and replicates were random factors. Means were compared based on Fisher's least significant difference.

3.2.5. Mating tests

Isolates of *Colletotrichum* spp. (Table 3.1) were used to perform mating experiments, and were regularly cultured on OMA as described above. *Lens culinaris* (lentil cv. Eston), *G. max*, *Phaseolus lunatus*, *T. pratense* and *Matricaria perforata* plants were grown and maintained in the greenhouse at 23°C - 25°C with a 16h photoperiod until senescence at which point the plants were harvested. The lignified stems were cut into equal pieces of 5 cm length and autoclaved at 121°C for 15 min.

Cross fertility was tested by mating all the *Colletotrichum* spp. isolates with the two cross fertile isolates of *C. lentis*, CT-21 (IG-2) and CT-30 (IG-1), and also with each other for isolates originating from the same host plant species. The cross between CT-

21 and CT-30 was used as a positive control in all experiments. All crosses were done using lentil stems and, wherever available, stems of the other respective host plant. Conidial suspensions were prepared by flooding 10 to 14 days old fungal cultures with sterile, distilled water, determining the spore concentration of suspensions using a haemocytometer and diluting them to obtain a final concentration of 2×10^5 spores mL^{-1}. The sterilized plant stems were inoculated for 2 hours in a mixture made up of a 5 mL spore suspension of the two isolates to be mated, or 10 mL of an individual isolate in the case of selfing. Five stems per Petri dish were placed on Whatman No. 1 filter paper in four replicate Petri dishes for each combination, sealed with parafilm and incubated in the dark at 22°C and 70% relative humidity for 10-14 days (Armstrong-Cho and Banniza, 2006). The presence of perithecia was monitored under a microscope (Nikon SMZ-U Zoom and Nikon MKII Fibre Optic Lamp, Nikon, NY), treating the formation of a minimum of one perithecium as a successful mating event.

3.3. Results

3.3.1. Phylogenetic Analysis

Sequence data of the six genes was concatenated to generate a supermatrix of 2186 characters. The topology of the Bayesian tree was similar to that obtained with maximum likelihood. The nucleotide sequences of all 19 isolates of *C. lentis* were identical for all six loci without any single-nucleotide polymorphisms (SNPs). *Colletotrichum lentis* grouped together in a unique cluster and belonged to the *destructivum* clade (Figure 3.1). This clade also included *C. linicola* (CLA01), *C. higginsianum* (IMI349063), *C. destructivum* (DAOM225582 - 225585) and the *C.*

truncatum isolate from *Matricaria perforata* (CT-64). The *truncatum* clade consisted of the epitype specimen (CBS151.35) and the second *C. truncatum* from *Phaseolus lunatus* (CBS119189). The *dematium* clade comprised the epitype specimen of *C. dematium* (CBS125.25) and three *C. truncatum* isolates from *Glycine max*. The *C. linicola, C. higginsianum*, *C. destructivum*, *C. dematium*, *C.truncatum* and *C. lindemuthianum* species were well resolved in the phylogenetic tree exhibiting high bootstrap and posterior probability support.


CT-21
CT-28
CT-30
CT-31
CT-32
CT-34
CT-35
CT-37
CT-39
CT-43
CT-58
CT-60
CT-233
GT-14
GT-60
GT-95
GT-150
CT-44
GT-155
Colletotrichum lentis
CT-64
C. truncatum ex Matricaria perforata
CLA01
C. linicola
IMI349063
C. higginsianum
DAOM225582
DAOM225583
DAOM225584
DAOM225585
C. destructivum
CT-49
C. truncatum ex Glycine max
CBS125.25
C. dematium
CT-53
CT-56
C. truncatum ex Glycine max
CBS151.35
CBS119189
C. truncatum ex Phaseolus lunatus
CLO2
C. lindemuthianum
99
60
99
81
99
54
99
99
68
99
99
0.05

(a)

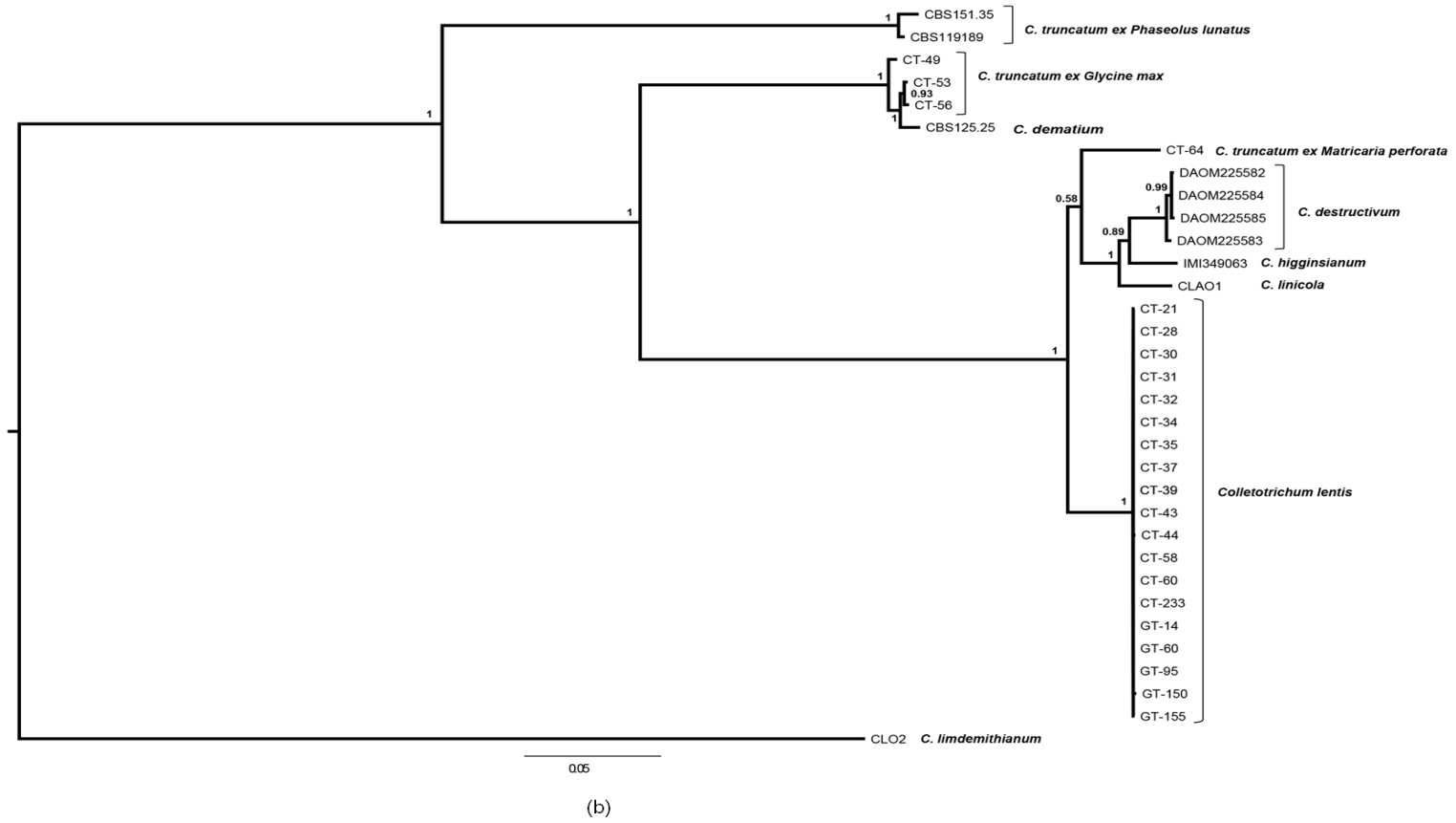


Fig. 3.1. Phylogenetic tree derived from ACT, β-TUB, CHS-1, GAPDH, HIS3 and ITS sequences of *Colletotrichum* spp. (a) Maximum likelihood analysis. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap analysis (1000 replicates) is shown next to the branches. (b) Bayesian Inference. A separate model of evolution for all genes was used to construct the tree. The values for Bayesian posterior probability (>0.5) are shown. *Colletotrichum lindemuthianum* was the outgroup used to root the phylogenetic tree in both analyses. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site (0.05).

3.3.2. Conidial Morphology

Conidia of *C. lentis* were slightly falcate with obtuse apices similar to those of *C. truncatum ex Matricaria perforata* except that they were more truncated, whereas the conidia of *C. destructivum* were cylindrical with rounded ends (Fig. 3.2 a-c). Conidial size of *C. lentis* (16-20 µm x 3-4 µm) and *C. truncatum ex Matricaria perforata* (15-20 µm x 4-5 µm) overlapped, whereas those of *C. destructivum* were smaller (8-12 µm x 4-5 µm) (Fig. 3.2 a-c). Conidia of *C. linicola*, *C. higginsianum* and *C. lindemuthianum* were cylindrical with dull, obtuse and ovoidal apices respectively (Fig. 3.2 d-f). *Colletotrichum lindemuthianum* conidia were much smaller (9-13 µm x 5-6) in comparison to *C. linicola* (10-18 µm x 3-5 µm), and *C. higginsianum* (14-17 µm x 3-4 µm). The epitypes of *C. truncatum* (20-28 µm x 3-5 µm) and *C. dematium* (20-25 µm x 3-4 µm) along with the *Colletotrichum* sp. isolate from *Glycine max* (20-23 µm x 3-5 µm) had conidia of lunate to falcate appearance and were longer compared to the other species (Fig. 3.2 g-i).

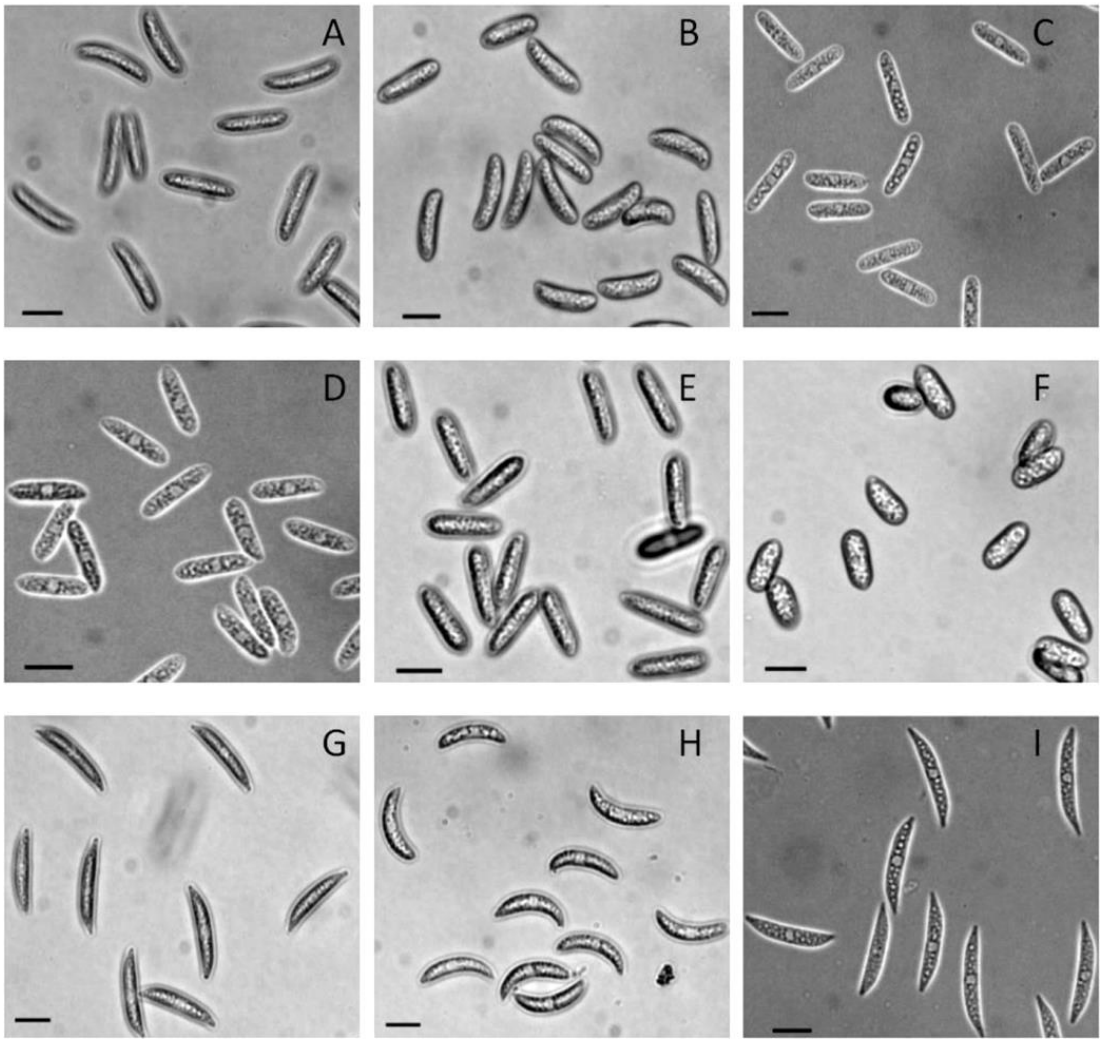


Figure 3.2. Conidia of *Colletotrichum* spp.: (A) *C. lentis*, (B) *C. truncatum* (*Matricaria perforata*, CT-64), (C) *C. destructivum* (DAOM225582), (D) *C. linicola*, (E) *C. higginsianum* (IMI349063), (F) *C. lindemuthianum*, (G) *C. dematium* (CBS125.25), (H) *C. truncatum* (CBS151.35) and (I) *C. truncatum* (*Glycine max*, CT-53) Bars = 10 µm.

3.3.3. Host Range

All isolates were able to infect and cause disease on their original host plant species included in this study, and usually induced significantly more disease on those than on other hosts (Table 3.2). Typical water-soaked anthracnose lesions that later became necrotic were seen on stems and leaves of infected plants, except for *A. thaliana* and *B. chinensis* inoculated with *C. higginsianum* on which only leaf lesions were observed (Figure 3.3). Leaf drop was observed for some host-isolate combinations, and in highly susceptible reactions, wilting of the entire plant was also observed. Non-host plant species did not display any lesions on either the leaves or stems.

Inoculation with isolates of *C. lentis* (race 0 and 1) resulted in 90% and higher disease severity on leaves of *L. culinaris* cv. Eston (both races), cv. CDC Robin (race 0) and *Pisum sativum* (both races), whereas it was significantly lower for CT-21 (race 1) on the partially resistant CDC Robin (19%) (Table 3.2). Moderate disease levels of 40 to 51% were observed on *V. faba* after inoculation with *C. lentis.* Disease severity levels of 43% were observed for *C. lindemuthianum* on *Phaseolus vulgaris*, whereas disease severity for other isolate-host combinations ranged from 5 to 26% (Table 3.2).

All isolates tested had similar pathogenicity on *Medicago truncatula* (5-11% disease severity on leaves). In addition, all isolates tested except for *C. lentis* were pathogenic on *Glycine max* (5-8% disease severity). Among the isolates of the *destructivum* clade, *C. linicola*, *C. higginsianum* and *C. lentis* all infected *L. culinaris* and *Pisum sativum*, and *C. linicola* and *C. lentis* were both pathogenic on *V. faba*. *Colletotrichum linicola* was also pathogenic on *C. arietinum*, and *Medicago sativa,* and *C. higginsianum* on *A.*

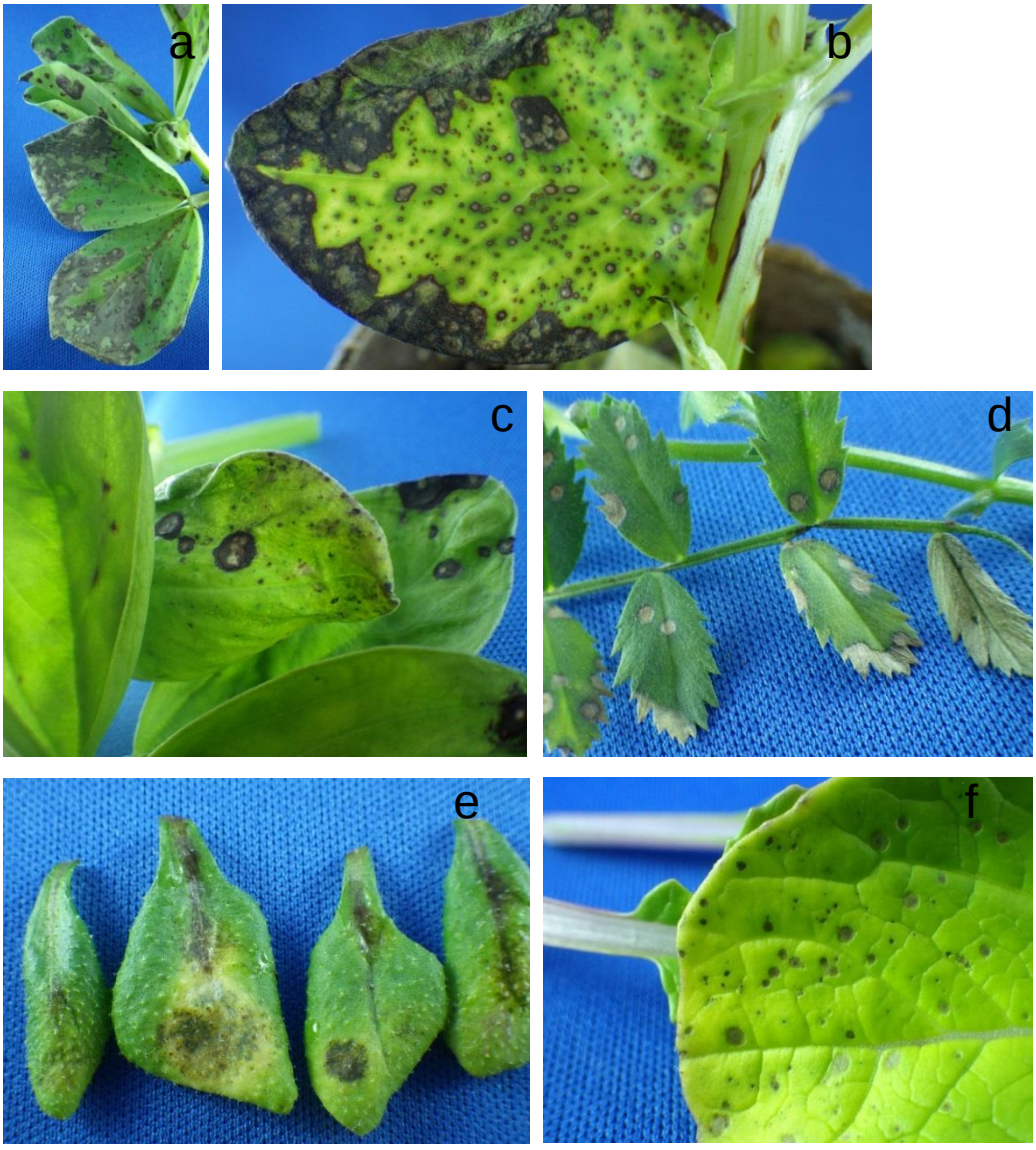
thaliana. *Colletotrichum destructivum* was pathogenic on *Pisum sativum*, as was *C. lentis*, and on *Medicago sativa* but not on *L. culinaris*. The epitype specimen of *C. truncatum* was pathogenic on *Pisum sativum*, *Glycine max*, *Phaseolus vulgaris*, *T. pratense* and *Medicago sativa*, but not on *L. culinaris*. The epitype specimen of *C. dematium* was pathogenic on *Glycine max*.

Table 3.2. Mean disease severity (%) on plant species (± standard error of the mean) inoculated with *Colletotrichum* spp. under controlled conditions. 0: no disease, - : not tested

Plant species	CT-21	CT-30	CLA01	IMI 349063	DAOM 225584	CT-53	CBS 151.35	CBS 125.25	CLO2
***Lens culinaris* cv. Eston**	91±2.6	95±0	9±1.8	6±1.3	0	0	0	0	0
***L. culinaris* cv. CDC Robin**	19±5.6	90±2.7	5±0	5±0	0	0	0	0	0
Pisum sativum	93±1.6	90±2.7	16±3.5	5±0	6±1.3	0	26±4.3	0	0
Cicer arietinum	0	0	6±1.3	0	0	0	0	0	0
Vicia faba	40±4.2	51±5.6	10±1.9	0	0	0	0	0	0
Glycine max	0	0	5±0	6±1.3	5±0	6±1.3	5±0	8±1.6	5±0
Phaseolus vulgaris	0	0	0	0	0	5±0	8±1.6	0	43±5.3
Phaseolus lunatus	0	0	0	0	0	5±0	11±1.8	0	6±1.3
Medicago sativa	0	0	8±1.6	0	5±0	0	6±1.3	0	0
Trifolium pratense	0	0	0	0	11±1.8	0	9±1.8	0	0
Medicago truncatula	5±0	10±1.9	9±1.8	11±2.6	6±1.3	6±1.3	9±1.8	9±1.8	5±0
Arabidopsis thaliana	0	0	0	5*	0	0	0	0	0
Brassica chinensis	-	-	-	25*	-	-	-	-	-

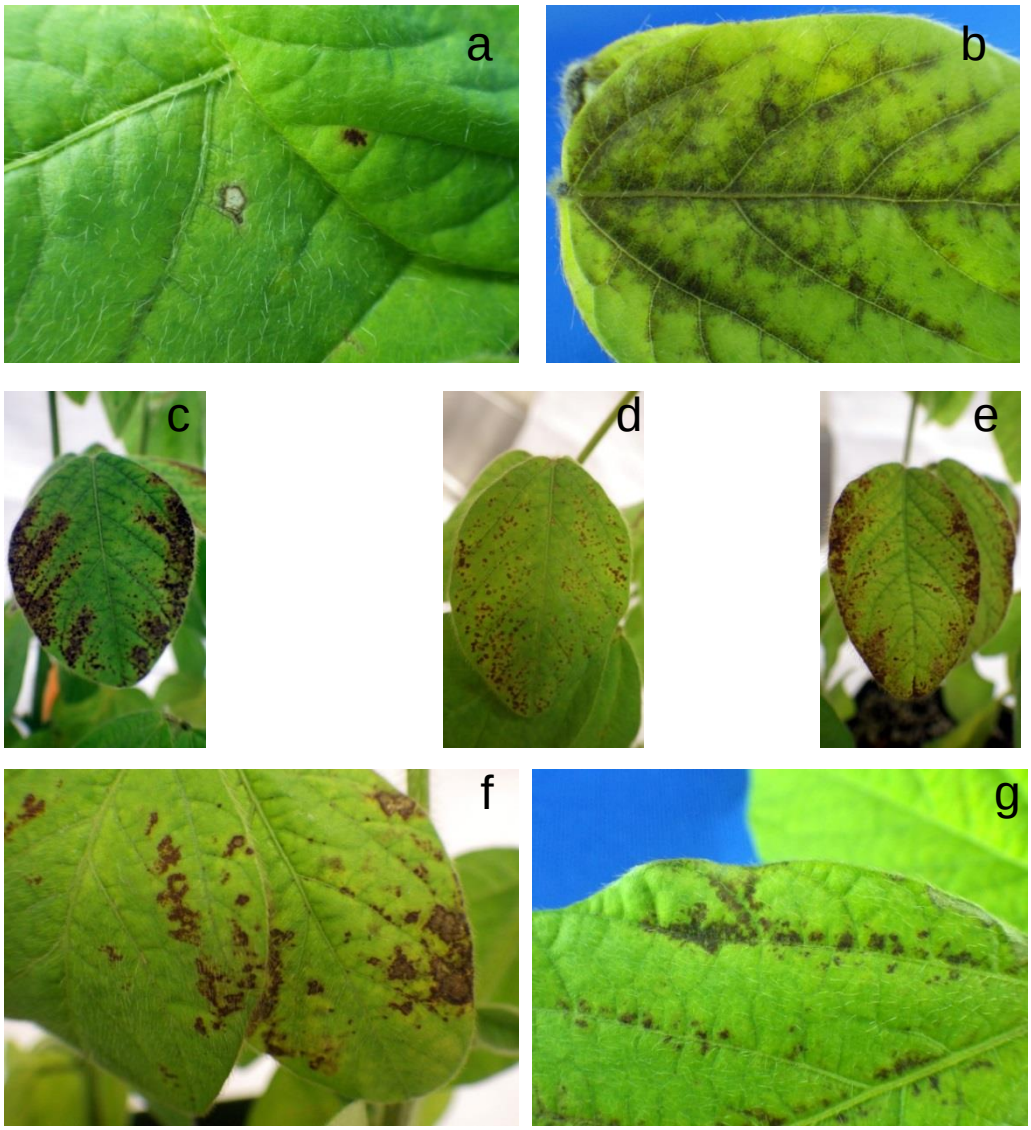
(A)

(B)



(C)

(D)



(E)

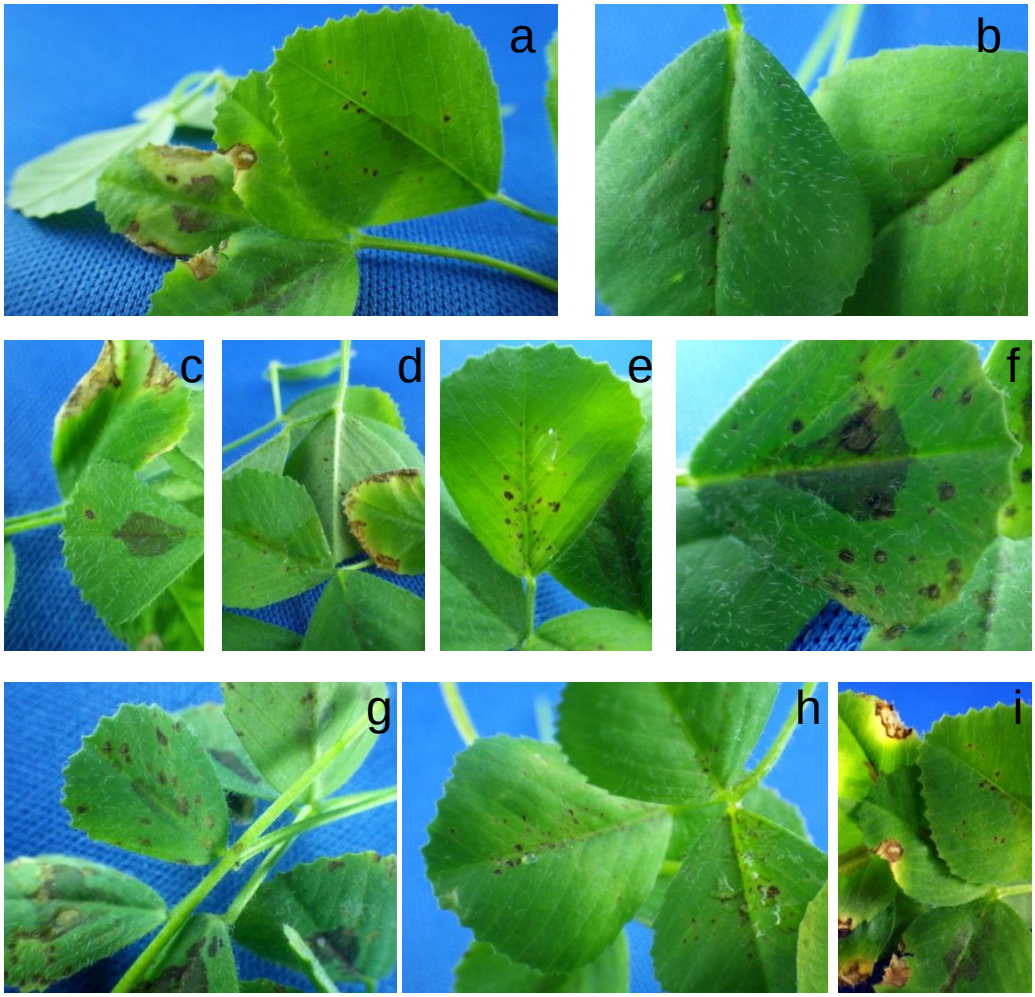


Fig. 3.3. Anthracnose symptoms on leguminous and brassicaceous plant species after inoculation with *Colletotrichum* spp. (**A**) *Lens culinaris* cv. Eston (a, d, g, j), *Lens culinaris* cv. CDC Robin (b, e, h, k) and P*isum sativum* (c, f, i, l) inoculated with CT-21 (a-c), CT-30 (d-f), IMI349063 (g-i) and CLAO1 (j-l). (**B**) *Vicia faba* (a, b, c), *Cicer arietinum* (d), *Arabidopsis thaliana* (e) and *Brassica chinensis* (f) inoculated with CT-21 (a), CT-30 (b), CLAO1 (c, d) and IMI349063 (e, f). (**C**) *Medicago sativa* (a, b), *Trifolium pratense* (c, d), *Pisum sativum* (e, f), *Phaseolus lunatus* (g, h, i) and *Phaseolus vulgaris*

(j, k, l) inoculated with CBS151.35 (a, c, e, g, j), DAOM225584 (b, d, f), CT-53 (h, k) and CLO2 (i, l). (**D**)*Glycine max* (a-g) inoculated with CT-53 (a), CBS151.35 (b), CBS125.25 (c), IMI349063 (d), DAOM225584 (e), CLAO1 (f) and CLO2 (g). (**E)** *Medicago truncatula* (a-i) inoculated with CT-21 (a), CT-30 (b), CT-53 (c), CBS151.35 (d), CBS125.25 (e), IMI349063 (f), CLAO1 (g), DAOM225584 (h) and CLO2 (i)

3.3.4. Classical mating

None of the isolates tested from *C. truncatum ex Phaseolus lunatus, C. truncatum ex Matricaria perforata*, *C. dematium ex E. campestre, C. higginsianum ex B. chinensis, C. linicola*, *C. destructivum ex T. pratense and Colletotrichum sp. ex G. max*, were cross fertile with the two *C. lentis* isolates CT-21 and CT-30 on lentil stems or on their host plant species (if used, Table 3.3). In contrast, perithecia were always observed for the cross between CT-21 and CT-30, in abundance on lentil stems but in slightly reduced number on stems of other plant species. All isolates of *Colletotrichum* spp. were self-sterile (Table 3.3). The crosses between isolates originating from the same host plant i.e. *T. pratense* (*C. destructivum*: DAOM225582, DAOM225583, DAOM225584 and DAOM225585), *Phaseolus lunatus* (*C. truncatum*: CBS151.35 and CBS119189) and *G. max* (*C. truncatum*: CT-49, CT-53 and CT-56), failed to produce perithecia irrespective of the stem material used.

Table 3.3. Mating of *Colletotrichum* spp. isolates with *Colletotrichum lentis* isolates on sterile lentil and respective available host plant stems.

Isolate	**Species**	**Mating Test**		
		CT-21(race 1)	**CT-30 (race 0)**	**Selfing**
CT-21	*C. lentis*	–	+	–
CT-30	*C. lentis*	+	–	–
CT-49	*C. truncatum*	–	–	–
CT-53	*C. truncatum*	–	–	–
CT-56	*C. truncatum*	–	–	–
CT-64	*C. truncatum*	–	–	–
CBS151.35	*C. truncatum*	–	–	–
CBS119189	*C. truncatum*	–	–	–
CBS125.25*	*C. dematium*	–	–	–
IMI349063*	*C. higginsianum*	–	–	–
DAOM225582	*C. destructivum*	–	–	–
DAOM225583	*C. destructivum*	–	–	–
DAOM225584	*C. destructivum*	–	–	–
DAOM225585	*C. destructivum*	–	–	–
CLAO1*	*C. linicola*	–	–	–

+: presence of perithecia, –: Absence of perithecia, *: host plant stem material not used.

3.4. Discussion

A combination of multi-locus phylogenetics, conidial morphology, *in vitro* mating and host range testing were used to determine the relationship between isolates of *C. lentis* from Western Canada that belong to two pathogenic races and two incompatibility groups and also to other closely related *Colletotrichum* species. Multi-locus phylogenetic analysis of several field and ascospore-derived isolates of *C. lentis* resulted in their grouping as a single cluster, exhibiting identical sequences at all 6 loci. Isolates of *C. lentis* as well as *C. linicola*, *C. higginsianum* and *C. truncatum ex Matricaria perforata* were part of the *destructivum* clade, but the latter three were more closely related to *C. destructivum* than to *C. lentis*. The three *Glycine max* isolates previously designated as *C. truncatum* were clustered in the *dematium clade* and closely related to the epitype of *C. dematium*. The epitype of *C. truncatum ex Phaseolus lunatus* was distinct from all other isolates previously identified as *C. truncatum*.

The presence of two pathogenic races (Buchwaldt et al. 2004) and two incompatibility groups (Menat et al. 2012) in the *C. lentis* population suggest a repeated introduction and/or a change in host, since it is unlikely that such a complex population structure would have evolved from a single ancestor in the short period in which this pathogen has been observed in Western Canada. This is particularly likely since the teleomorph appears to be absent in nature. The fact that resistance to one of the races was found in species other than *Lens culinaris* led to the hypothesis that the two races may represent distinct taxa, but results here confirmed that isolates of both races belong to the same species and do not represent a mixture of two species. The ascomycete *Leptosphaeria*

maculans was originally described as a species consisting of a weakly virulent B group and highly virulent A group, until morphological (Shoemaker and Brun, 2001) and ITS phylogeny studies (Mendes-Pereira et al. 2003) revealed that only the highly virulent isolates belonged to *L. maculans* whereas the weakly virulent isolates belonged to *L. biglobosa*. Several previous studies involving isolates of *C. lentis* have been conducted to determine genetic similarities and examine the phylogeny of closely related *Colletotrichum* spp. Although there was evidence for the distinctiveness of isolates from *L. culinaris*, the lack of a type or epitype specimen for *C. truncatum* prevented the clarification of their taxonomy until recently (Damm et al. 2009). Ford et al. (2004) reported that 19 *L. culinaris* isolates from Western Canada were genetically very similar (99.8-100%) based on their ribosomal DNA sequences, but RAPD analysis revealed higher genetic diversity (0-16%) among them. Lack of polymorphisms in the 5.8s rDNA-ITS of *L. culinaris* isolates, and their distinctiveness from *C. truncatum* isolates from *Matricaria perforata* were reported earlier by Forseille et al. (2011). The employment of rDNA sequences and examination of infection strategies indicated that *L. culinaris* isolates were phylogenetically closely related to *C. destructivum* and *C. linicola* (Latunde-Dada and Lucas 2007). The recent description of an epitype specimen of *C. truncatum* from *Phaseolus lunatus* (Damm et al. 2009) enabled the comparison of the epitype with putative *C. truncatum* isolates and suggested that the *L. culinaris* isolates belong to the *destructivum* clade (O'Connell et al. 2012), which is in agreement with results of the *destructivum* clade phylogeny (Damm et al. 2014).

Data of conidial shape and size allowed *C. lentis* to be distinguished from the epitype specimen of *C. truncatum* from *Phaseolus lunatus*, *C. truncatum* from *Glycine max* and from *C. destructivum*. However, conidial dimensions could not clearly distinguish *C. lentis* from *C. higginsianum*, *C. linicola* and *C. truncatum* from *Matricaria perforata*. This confirms observations by Cai *et al*. (2009) who previously noted that conidial shape and size may not be sufficient for differentiation among certain *Colletotrichum* species, but can lead to a better resolution of species if used in combination with molecular data and host range studies. The mating experiments between *C. lentis* and closely related *Colletotrichum* spp. to test for intra- and inter-specific fertility following the biological species concept revealed no successful interspecific matings. Results suggest that in the genus *Colletotrichum* interspecies mating does not occur, in contrast to the genus *Ascochyta*, where *in vitro* crosses between host specific and phylogenetically distinct isolates of *A. fabae* and *A. lentis* successfully produced viable progeny (Kaiser et al. 1997). However, intraspecific mating experiments of isolates of *C. destructivum*, *C. truncatum ex Phaseolus lunatus* and *C. truncatum ex Glycine max* on their respective host plant stems also failed to demonstrate any self or cross fertility. Although not surprising considering that *Colletotrichum* is known to be a predominantly asexual genus (Crouch et al. 2014), and considering that a high level of variation in fertility has been reported for cross-fertile *C. lentis* isolates as well (Menat et al. 2012), more isolates of species other than *C. lentis* used in this study would need to be tested to conclusively determine intra- and interspecific fertility.

The host range of the *C. lentis* observed in this study agreed with previous reports (Gossen et. al 2009), with the addition of *Medicago truncatula*. The isolates of *C. truncatum* from *Matricaria perforata* were not included in this study as they had been previously assessed for host range and shown to cause limited lesions on *L. culinaris*, *C. arietinum*, *Pisum sativum* and *V. faba* (Gossen et al. 2009).*Colletotrichum linicola* and *C. higginsianum* have host ranges that overlap with that of *C. lentis* and include *L. culinaris* as a common host. In contrast, *C. destructivum* and the epitype of *C. truncatum* have fewer host plants in common with *C. lentis*, and do not infect the two closely related host species *L. culinaris* and *V. faba* (Doyle and Luckow 2003). Host ranges of *Colletotrichum* spp. used in this study has been extensively reviewed previously, except for those newly reported here (Hyde et al. 2009; Canon et al. 2012). The fact that many of the isolates used for the host range study were pathogenic on numerous hosts confirmed that *Colletotrichum* species are not highly host-specific pathogens (Freeman et al. 1998). Host range tests revealed significant differences in the amount of disease caused by different isolates. Isolates of *C. lentis* and *C. lindemuthianum* caused significantly more disease on their host of origin, and in the case of *C. lentis* on *Pisum sativum* and *V*. *faba*, compared to the other *Colletotrichum* spp. tested. This can likely be attributed to higher levels of resistance in some of the cultivars used in this study that had been selected without prior knowledge of their resistance except for *L. culinaris*. As an example, lower disease severity on *L. culinaris* cv. CDC Robin was observed after inoculation with CT21 (race 1) because this cultivar possesses partial resistance to this race. Co-evolution between the field isolates of *C. lentis* and *C. lindemuthianum* and their respective hosts that have been bred for

improved anthracnose resistance may also have increased the virulence of these pathogens compared to the isolates from host species that are not field crops. Suboptimal incubation conditions for some isolate-host combinations may also have contributed to lower disease development, as observed for *C. higginsianum* (IMI349063) which required high humidity for 96 h instead of 48 h for successful infection of *A. thaliana*.

In conclusion, the *C. lentis* population from Western Canada was confirmed to represent one distinct taxon. All isolates of *C. lentis* displayed 100% sequence identity for the six gene regions examined, thus rejecting the hypothesis of a mixed species population. Conidial morphology was shown to be effective in discriminating between *C. lentis* and the epitype specimen of *C. truncatum*, but an overlap in conidial dimensions was seen among taxa in the *destructivum* clade. The ability of *C. higginsianum* and *C. linicola* to cause anthracnose on *L. culinaris*, and the inability of either the epitype of *C. truncatum* or *C. destructivum* to infect *L. culinaris*, supports their respective relationships to *C. lentis* based on molecular data. All *Colletotrichum* spp. tested here infected the model plant species *Medicago truncatula* which could be of interest for comparative infection and host-pathogen interaction studies among the different species of *Colletotrichum*. This may allow for the wealth of molecular information already generated on host-pathogen interactions in *Medicago truncatula* to be exploited for resistance breeding in field crops such as *L. culinaris*, *Pisum sativum* and *V. faba* based on synteny among their genomes (Sharpe et al. 2013).

3.5. Prologue to Chapter 4

The aim of this study was to develop a better understanding of the genetic regulation of the mating process in *C. lentis*. Previous studies have suggested that the mating system in *C. lentis* is atypical of heterothallic ascomycete fungi and is characterized by unbalanced heterothallism. Hence in the following research chapter the mating type gene (*MAT1-2*) of *C. lentis* isolates from Chapter 3 were characterized, the expression profile was determined and the possibility of splicing events of *MAT1-2* in isolates CT-21 (IG-2) and CT-30 (IG-1), the mating pair used for performing mating tests in Chapter 3, was assessed.

CHAPTER4

EXAMINATION OF THE MATING TYPE GENE (*MAT1-2*) OF *COLLETOTRICHUM LENTIS* FROM LENTIL IN WESTERN CANADA

4.1. Introduction

Mating in filamentous ascomycetes is a very intricate process initiated via a pheromone signal between sexually compatible haploid cells and governed by the mating type (MAT) locus. The mating type locus of filamentous fungi was first characterized in *Saccharomyces cerevisiae* (Astell et al. 1981), followed by cloning of the genes present at the MAT locus for *Neurospora crassa* (Glass et al. 1988).

The MAT locus is a distinct part of the fungal genome as it contains dissimilar DNA sequences between opposite mating type cells, termed *MAT1-1* and *MAT1-2*, which are referred to as idiomorphs. The MAT locus is involved in identifying cell-type and choreographing the sexual cycle in fungi, and plays important roles in the development of sexual structures, gametes and post-fertilization events. The number of genes present at the MAT locus of the idiomorphs generally ranges from one to three, and encode DNA binding transcriptional factors, namely the α-box domain and the high mobility group (HMG) domain. Ascomycetes can be designated as either homothallic or heterothallic. In heterothallic ascomycetes, the partners involved in a fertile mating event must be of opposite mating types, carrying either *MAT1-1* or *MAT1-2* at the MAT locus (Kronstad and Staben, 1997). In contrast, a homothallic individual contains both idiomorphs and is self-fertile (Poggeler, 2001).

Members of the genus *Colletotrichum* appear to predominantly reproduce asexually, evident from the fact that the sexual state in the genus has been described in only a few species (Crouch et al. 2014). On the other hand, classical mating experiments in the laboratory under controlled conditions have been successfully performed in *C. graminicola*, *C. lindemuthianum* and *C. lentis,* whose sexual morphs have not been observed in nature (Vaillancourt and Hanau, 1991; Rodriguez et al. 2005; Armstrong-Cho and Banniza, 2006). The mating system in *Colletotrichum* is puzzling as it deviates from the typical bipolar mating system of the ascomycetes fungi regulated by the two idiomorphs at one locus. For instance, mating in *Colletotrichum gloeosporioides* is considered to be of a complex nature involving more than a single MAT locus that contains more than two alleles (Wheeler, 1954), but a single locus with multiple alleles at the *MAT1-2* has also been proposed (Cisar and TeBeest, 1999). Genetic studies of heterothallism in *G. graminicola* suggested that two unlinked loci govern mating (Vaillancourt et al. 2000).

In all *Colletotrichum* spp. studied so far, whether homothallic or heterothallic, only the conserved HMG DNA binding domain of the *MAT1-2* idiomorph has been observed and all efforts to detect the α-box domain of the *MAT1-1* idiomorph have proven futile, suggesting that it does not exist in the *Colletotrichum* genome (Vaillancourt et al. 2000; Rodriguez et al. 2005, Menat et al. 2012). In order to explain the unconventional mating, the theory of ‘unbalanced heterothallism’ was proposed by Wheeler (1954) for the genus *Colletotrichum* that assumes the genus to be homothallic, but that a mutation in mating genes inhibits the sexual cycle. The coming together of isolates carrying

different mutations can enable the sexual cycle via complementation, resulting in a heterothallic mating behavior. It was also suggested that sexual reproduction in the genus is distinct from either the homothallic or heterothallic system reported in filamentous ascomycetes (Vaillancourt et al. 2000).

The sexual morph of *C. lentis* from lentil has not been observed in the field to date, but was successfully induced in the laboratory by incubating *C. lentis* isolates on lentil stems on distilled water agar media (DWA) (Armstrong-Cho and Banniza, 2006). Further classical mating studies on 21 isolates of *C. lentis* resulted in the separation of isolates into two incompatibility groups, IG-1and IG-2, with isolates only displaying cross-fertility with members of the opposite group (Menat et al. 2012). This strongly supports a heterothallic, bipolar mating system in this species, and agrees with the typical mating system of heterothallic filamentous ascomycetes. The production of perithecia was highly variable, similar to earlier reports in *C. graminicola* (Vaillancourt and Hanau, 1991), and a few crosses were sterile, which was attributed to a low level of fertility below the detection level. However, only the *MAT1-2*, and not the *MAT1-1* idiomorph was present in isolates of both incompatibility groups (Menat et al. 2012).

The *MAT1-2* gene has been sequenced and characterized in a few *Colletotrichum* species, such as *C. lindemuthianum* (Garcia-Serrano et al. 2008)*, C. gloeosporioides* (Silva et al. 2012)*, C. fructicola* (Silva et al. 2012), *C. orbiculare* (Gan et al. 2012), *C. graminicola* and *C. higginsianum* (O'Connell et al. 2012). The *MAT1-2* gene of all *Colletotrichum* spp. contains the conserved HMG-box, but the *Mat1-2* transcript varies in length: *C. higginsianum* (987 bp), *C. lindemuthianum* (870 bp), *C. graminicola* (840

bp), *C. orbiculare* (750 bp) and *C. fructicola* (726 bp). A high degree of interspecific diversity is also present at the *MAT1-2* locus. The nucleotide sequences of *C. orbiculare* and *C. fructicola* differ by 34% and both share just over 50% nucleotide sequence similarity with the *MAT1-2* of *C. graminicola*. The *MAT1-2* of *C. higginsianum* is the most divergent displaying around 70% sequence dissimilarity to *C*. *graminicola*, *C*. *orbiculare* and *C*. *fructicola* (Crouch et al. 2014). The diversity evident among *MAT1-2* genes has also been employed to interpret and clarify phylogenetic relationships of *Colletotrichum* spp. (Du et al. 2005). Despite the interspecific diversity in the *MAT1-2* gene, it is reported that the *MAT* locus in the *Colletotrichum* species analyzed so far is completely conserved in terms of gene content and gene order (reviewed by Crouch et al. 2014). At the intraspecific level, the *MAT1-2* nucleotide sequences are almost identical, e.g. 99 - 100% sequence identity was observed between sexually compatible partners in *C. lindemuthianum* (Garcia-Serrano et al. 2008).

The objectives of this study were to (1) sequence and characterize the *MAT1-2* gene of *C. lentis* isolates from both incompatibility groups (IG-1 & 1G2), (2) study the expression profile of the *MAT1-2* gene in *C. lentis* and (3) investigate the possibility of alternative splicing in the *MAT1-2* gene of *C. lentis*.

4.2. Materials and methods

4.2.1. PCR amplification and sequencing of *MAT1-2*

All isolates of *C. lentis* (Table 3.1) were grown and maintained, and genomic DNA was extracted as previously described. The high mobility group (HMG) sequence information (Menat et al. 2012) for CT-21 and CT-30 was used to carry out a BLAST search against

the available sequence scaffold of the *C. lentis* genome (S. Banniza, unpublished data). All similar contig/s were aligned with the HMG sequences of CT-21 and CT-30 in MEGA 5.2 to determine sequences upstream and downstream. Using the gene prediction software FGENESH (Salamov and Solovyev, 2000), the putative *MAT1-2* gene sequence was determined. The identity of the gene was further validated by comparing it to the *C*. *higginsianum* and *C*. *graminicola* genome databases available within the Broad Institute database (http://www.broadinstitute.org/).

For amplification of the entire *MAT1-2* forward primer MAT-121F (5' - ACGATTTCCTTGACGAACACA - 3') and reverse primer MAT-121R (5' - TGCGTTATCTTGCTTCCCCC - 3') primers were designed using Primer 3 (Koressaar and Remm, 2007; Untergasser et al. 2012.) and NCBI Primer BLAST (Ye et al. 2012) software tools.. A 50 µl PCR reaction consisted of 10 ng genomic DNA, 1x PCR buffer (Invitrogen, Carlsbad, CA), 2 mM $MgCl_2$, 20 µM of dNTP, 0.2 µM of forward and reverse primers and 1 U of Taq DNA Polymerase (Invitrogen, Carlsbad, CA). The PCR cycling conditions included an initial denaturation step of 5 min at 94°C followed by 34 cycles at 94°C for 30 s, 52°C for 30 s and 72°C for 60 s, and a final elongation step at 72°C for 7 min.

The PCR amplicons were extracted, purified and sequenced as previously described (3.2.2). The *MAT1-2* gene sequences of all 19 *C. lentis* isolates were aligned, manually edited in MEGA 5.2 using the CLUSTALW program and were compared by constructing a multiple sequence alignment to detect any nucleotide variations between members of the incompatibility groups IG-1 and IG-2.

4.2.2. RNA extraction and first strand cDNA synthesis

Colletotrichum lentis isolates CT-21 (IG-2), CT-30 (IG-1) and a co-culture of CT-21 and CT-30 were grown for 7-10 days on OMA as described in Chapter 3. The culture plates were flooded with sterilized deionized water and a conidial suspension of 2 x 10^5 spores mL^{-1} was prepared for CT-21, CT-30 and the co-culture of CT-21 and CT-30. An aliquot of 1 mL from each of the three spore suspensions was added to 50 mL of glucose yeast medium (GYM) as described in Chapter 3 in conical flasks and incubated on a rotary shaker at 21-23°C and 130 rpm for 0h, 6h, 12h, 18h, 24h, 36h and 48h. At the end of each incubation time flasks containing CT-21, CT-30 and co-culture of CT-21 and CT-30 were twice subjected to centrifugation at 3,000 rpm for 5 min and the resulting pellet was immediately flash frozen in liquid nitrogen and stored at -80°C until further use.

Total fungal RNA was isolated using the TRIzol® reagent protocol (Life Technologies Inc. Burlington, ON). Fungal tissue weighing approximately 50-100 mg was ground to a fine powder in liquid nitrogen using a mortar and pestle, followed by the addition of 1 mL TRIzol® reagent to each sample. The samples were incubated for 5 min at room temperature after which 0.2 mL of chloroform was added. The tubes were shaken vigorously for 15 seconds and further incubated for 3 min at room temperature. The samples were centrifuged at 12,000 x g for 15 min at 4°C, the aqueous phase transferred to a new tube to which 0.5 mL of 100% isopropanol was added. The samples were then incubated for 10 min at room temperature. A second centrifugation at 12,000 x g for 15 min at 4°C was performed and the supernatant discarded leaving only the RNA pellet. The pellet was washed with 1 mL of 75% ethanol, briefly vortexed

and then centrifuged at 7500 x g for 5 min at 4°C with the supernatant being discarded and the pellet air-dried for 10 min. The RNA pellet was resuspended in 30 µL of DEPC-treated water (Life Technologies Inc. Burlington, ON) and incubated at 60°C for 15 min before being used for downstream applications. RNA was extracted for three independent biological replications.

The quality of the RNA samples was determined using the A_{260}:A_{280} ratio (~ 2) on a Nanodrop 8000 spectrophotometer (Thermo Fisher Scientific, Ottawa, ON) and the RNA integrity of the samples was checked by subjecting the samples to electrophoresis on a 1.2% (w/v) denaturing agarose gel containing formaldehyde in 1 x 3-(N-morpholino) propanesulfonic acid (MOPS) buffer (pH 7.0). An equal volume of 2X RNA loading dye (Thermo Fisher Scientific, Ottawa, ON) was added to each sample prior to loading on gel and the sharpness of the rRNA bands and a 2:1 ratio of 28S rRNA to 18S rRNA was observed.

Traces of any residual fungal genomic DNA were removed by treating the RNA samples with amplification grade DNase I (Invitrogen, Carlsbad, CA), following the manufacturer's protocol. A total of 2 µg RNA was used as template to perform first strand cDNA synthesis with the help of reverse transcriptase enzyme SuperScript™ III RT (Invitrogen, Carlsbad, CA), following the manufacturer's protocol. Non-DNase and non-RT treated samples were used as controls in the semi-quantitative expression profiling experiments.

4.2.3. Semi-quantitative reverse transcriptase PCR and alternative splicing

A semi-quantitative reverse transcriptase PCR approach was used to study the expression levels of *MAT1-2* in *C. lentis* isolates CT-21, CT-30 and a co-culture of CT-21 and CT-30 at seven *in vitro* time points, 0h, 6h, 12, 18h, 24h, 36h and 48h after inoculation in glucose yeast media. The cDNA obtained from the above steps was diluted 10X using sterile distilled water and used as template for the PCR reactions performed on the Bio-Rad S1000^{TM} thermal cycler. Specific forward and reverse primer pairs, HMGF1 (5'- CCGCACATCCAAAACCATGA - 3') / HMGR1 (5'- AGGGCTTGCGGGGAGAATAC-3') and ACTF1 (5'-GGCACAGTCGAAGCGTGGTA-3') / ACTR1 (5'- ACGGCCTGGATGGAGACGTA- 3') were designed as described earlier, to amplify the HMG domain of the *MAT1-2* gene and the *ACTIN* gene of *C. lentis*, respectively. The *ACTIN* gene was used as the internal control in the expression analysis, to compare the intensity of gel bands between samples. For the alternative splicing study the *MAT1-2* gene transcripts of isolates CT-21 and CT-30 were amplified using primer pair MAT-121F and MAT-121R (described above).

The 20 µl PCR mix comprised of 200 ng of cDNA, 1x PCR buffer (Invitrogen, Carlsbad, CA), 2 mM $MgCl_2$ (Invitrogen, Carlsbad, CA), 10 mM of dNTP mix (Invitrogen, Carlsbad, CA), 0.2 µM of forward and reverse primer and 1 U of Taq DNA Polymerase (Invitrogen, Carlsbad, CA). The PCR cycling conditions for semi-quantitative expression analysis of the *MAT1-2* gene consisted of 5 min at 94°C for denaturation followed by 29 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 60 s, with a final elongation at 72°C for 7 min. PCR cycling conditions for the alternative splicing study consisted of an initial

denaturation for 5 min at 94°C followed by 34 cycles at 94°C for 30 s, 52°C for 30 s and 72°C for 60 s, and a final elongation step at 72°C for 7 min. The resulting PCR products were visualized under UV light on a 1.5% (w/v) agarose gel stained with ethidium bromide and run in 1× TAE buffer for 1h at 100V. Data for the *MAT1-2* expression analysis and alternative splicing study was obtained from three independent biological and three technical replicates. The expression of *MAT1-2* was highly similar across all the replications and Fig.4.2 represents observations from one of the replication.

4.3. Results

4.3.1. *MAT1-2* of *Colletotrichum lentis*

A 1.1-kb sequence comprising *MAT1-2* was obtained for all 19 isolates of *C. lentis* belonging to both incompatibility groups (Fig. 4.1). *MAT1-2* consisted of two introns and three exons with an open reading frame (ORF) of 726 bp coding for a putative protein of 241 amino acids, including the conserved high mobility group (HMG) domain. The *MAT1-2* nucleotide sequences of all 19 *C. lentis* isolates were identical irrespective of IG. A nucleotide BLAST (blastn) query search of the *C. lentis MAT1-2* nucleotide sequence with available *MAT1-2* sequences of *Colletotrichum* spp. in GenBank and the Broad Institute database confirmed the identity of the *MAT1-2* gene sequenced. A Clustal W comparison for the *MAT1-2* nucleotide and the putative HMG box protein sequence of *C. lentis* isolates with the *MAT1-2* sequences of *C. higginsianum* (IMI349063) and *C.graminicola* (M1.001) (Broad Institute database), indicated a 93% and 75% sequence similarity, respectively.

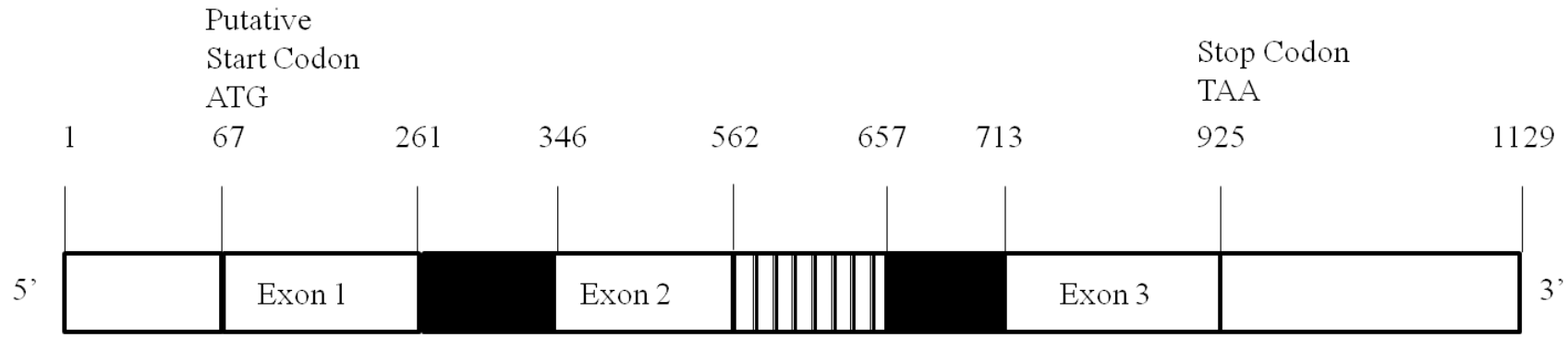


Fig. 4.1. Structure of the mating type gene, *MAT1-2*, of *Colletotrichum lentis*. Open boxes represent the exons, darkened boxes the introns and the shaded region represents the HMG-box. Numbers depict nucleotide base pairs.

4.3.2. Expression profiling and alternative splicing of *MAT1-2*

The transcriptional activity of *MAT1-2* was studied at different *in vitro* time points of *C. lentis*, along with the reference *ACTIN*. Based on semi-quantitative reverse transcriptase PCR expression profiles, *MAT1-2* of CT-21, CT-30 and the co-culture of CT-21 and CT-30 are expressed in a constitutive manner as the level of expression is similar from 0h to 48h of incubation (Fig. 4.2). The investigation for possible alternative splicing events for the *MAT1-2* transcript of *C. lentis* on the basis of variation in the base pair (bp) size revealed no differences in the transcript size between isolates CT-21 and CT-30 (Fig. 4.3).

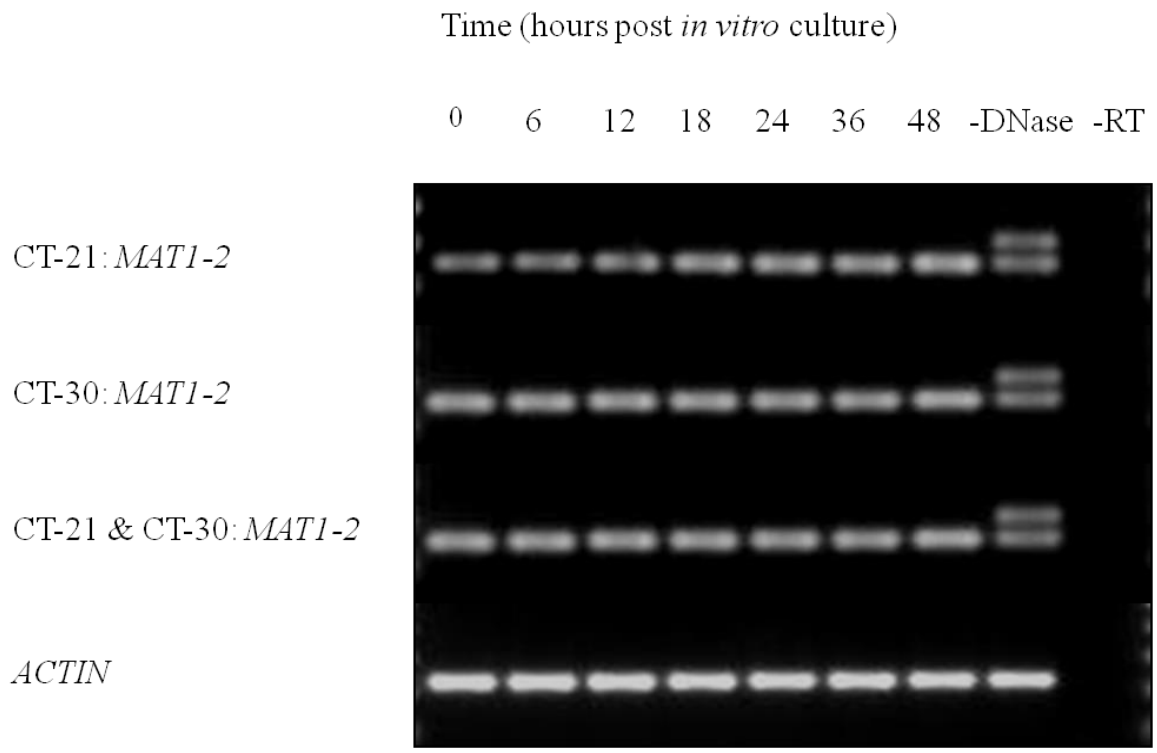


Fig. 4.2. Semi-quantitative RT-PCR expression profiles for *Colletotrichum lentis* transcripts of isolates, CT-21, CT-30 and co-culture of CT-21 & CT-30, encoding the mating type (*MAT1-2*) and actin, during *in vitro* growth at time points of 0, 6, 12, 18, 24, 36 and 48h post-culturing in liquid glucose yeast growth medium (GYM). Actin was used as the reference gene. A no DNase and no RT sample were included as controls for absence of genomic DNA contamination in samples.

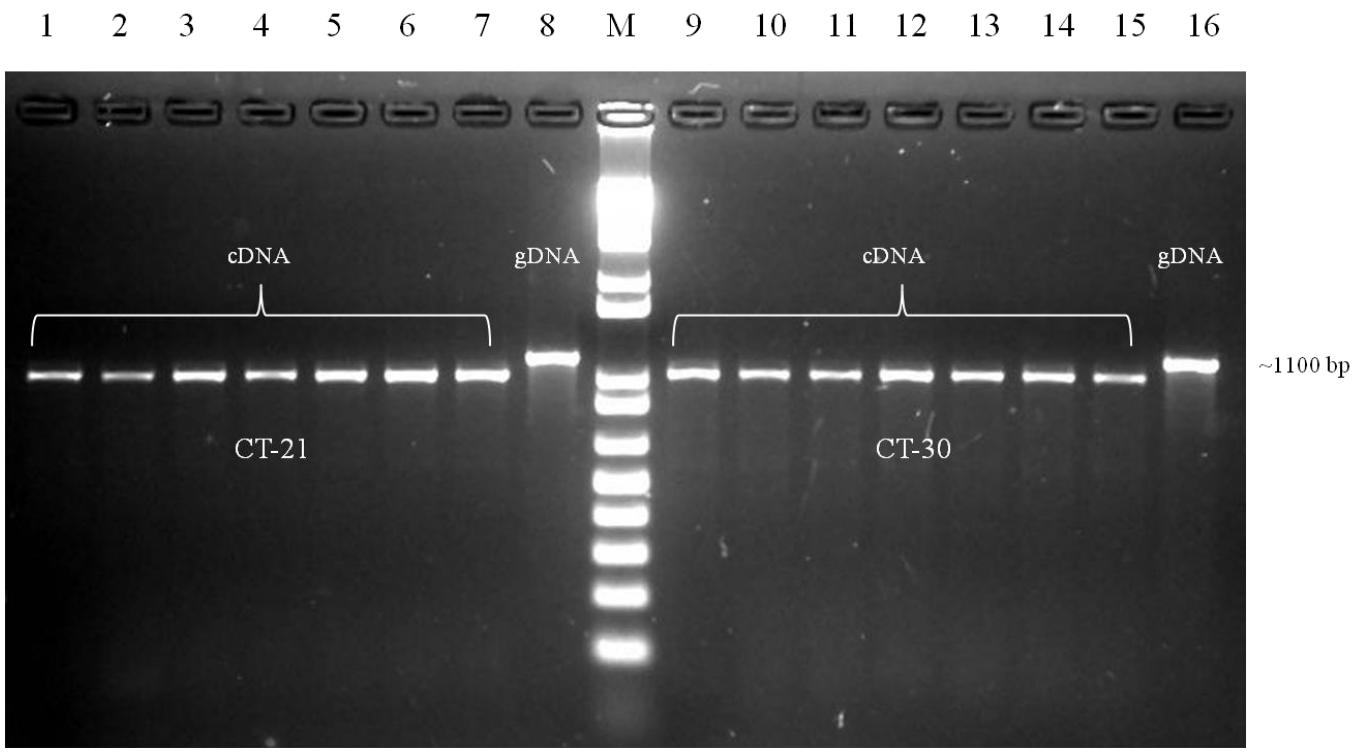


Fig. 4.3. Agarose gel electrophoresis of amplified *MAT1-2* transcripts of *Colletotrichum lentis* isolates CT-21 and CT-30 to investigate the possibility of alternative splicing events. Lanes 1-7 represent PCR amplicons from cDNA samples of CT-21 and lanes 9-15 of CT-30, obtained at 0, 6, 12, 18, 24, 36 and 48h post-culturing in GYM. Lanes 8 and 16 are PCR amplicons from genomic DNA of CT-21 (lane 8) and CT-30 (lane 16) used as controls. M: 1Kb plus DNA ladder.

4.4. Discussion

To better understand the unusual regulation of mating in *C. lentis*, the *MAT1-2* mating type gene of the *MAT1-2* idiomorph from 19 isolates of both incompatibility groups (IG-1/IG-2) was sequenced. Complete sequence identity for *MAT1-2* across all 19 isolates of *C. lentis* was observed, similar to the observations made in *C. graminicola* where both parents of a mating cross possessed identical copies of the *MAT1-2* gene (Vaillancourt et al. 2000). A low level of intraspecific variation within *MAT1-2* has been described in other ascomycete fungi (Turgeon, 1998). The sequence identity of *MAT1-2* in *C. lentis* eliminates the possibility of the involvement of any mutation or recombination of *MAT1-2* in determining incompatibility. This suggests that if the unbalanced heterothallism theory proposed by Wheeler (1954) for the unusual mating behaviour in members of *Colletotrichum* holds true, then mutations in genes other than the *MAT1-2* in *C. lentis* must be responsible for regulating the process of mating.

It was previously reported that the conserved domains characteristic of both mating type genes in *C. lentis* resulted in the amplification of the HMG domain in both mating partners, whereas the α1 box was not detected in any isolate tested (Menat et al, 2012). Similar observations have been made in *C.* graminicola, *C. lindemuthianum* and *C. higginsianum* (Vaillancourt et al. 2000; Rodriguez-Guerra et al. 2005; O'Connell et al. 2012). The occurrence of the *MAT1-2* idiomorph in both mating partners could be attributed to the presence of multiple copies of the *MAT1-2* gene in the *C. lentis* genome which could be clarified once the complete genome sequence of *C. lentis* is available. The study of *MAT1-2* gene copy number in *C. lindemuthianum* (Garcia-Serrano et al.

2008) using Southern hybridization, and in C. *graminicola* and *C. higginsianum* (O'Connell et al. 2012) through whole genome sequencing revealed that a single *MAT1-2* gene existed in each genome.

A comparison between the *MAT1-2* of *C. higginsianum* (O'Connell et al. 2012) and *MAT1-2* of *C. lentis* in the current study revealed a high level of similarity in the gene structure. The *MAT1-2* of *C. lentis* was composed of three exons and two introns similar to the *MAT1-2* reported in *C. higginsianum*, but different from the *MAT1-2* of *C. lindemuthianum* (Garcia-Serrano et al. 2008) which contains four exons and three introns. A 93% similarity observed for the putative protein sequence encoded by *MAT1-2* of *C. lentis* to *C. higginsianum* indicates that the predicted amino acid sequence for *C. lentis MAT1-2* seems accurate. It should also be noted that *C. higginsianum* and *C. lentis* are both part of the *C. destructivum* species complex and closely related based on phylogenetic analysis (Fig. 3.1; O'Connell et al. 2012; Damm et al. 2014) suggesting that the *MAT1-2* gene is potentially conserved in structure and protein sequence in members of this clade.

The semi-quantitative RT-PCR expression profiling of *MAT1-2* in *C. lentis* using mating pair isolates CT-21 and CT-30 and a co-culture of CT-21 and CT-30 demonstrated a constitutive expression of *MAT1-2*. This indicated that the gene is functional and its expression is independent of the fungal growth stage and conditions (cultured separately or co-cultured). The observation that *MAT1-2* of *C. lentis* is transcribed in vegetative mycelia could be ascribed to the theory of mating type genes being involved in non-mating activities such as vegetative incompatibility, sexual dimorphism and

virulence (Kronstad and Staben, 1997, Bidard et al. 2011). As only the *MAT1-2* has been detected in all *Colletotrichum* spp. examined so far, the hypothesis was tested here that mutations in *MAT1-2* of *C. lentis* due to alternative splicing, a common occurrence in eukaryotes, could affect gene expression and result in synthesis of a new protein isoform. The lack of variation in the *MAT1-2* transcript size between CT-21 and CT-30 when observed by agarose gel electrophoresis indicates that *MAT1-2* of *C. lentis* does not undergo alternative splicing. Based on the *Neurospora crassa* genome, it has been inferred that alternative splicing, although present, occurs at a low rate in ascomycete fungi (Kempken, 2013). Functional analysis of the *MAT1-2* in *C. lentis* needs to be performed in future to determine not only its downstream target genes that remain unknown, but also to achieve a better understanding of the genetic regulation employed by mating type genes in *C. lentis.*

In conclusion, the *MAT1-2* of *C. lentis* displays sequence identity among isolates from both incompatibility groups indicating that gene(s) other than *MAT1-2* are responsible for the regulation of sexual incompatibility in *C. lentis*. Also the *MAT1-2* is constitutively expressed in *C. lentis* with no evidence for alternative splicing. A detailed understanding of the mating system of *C. lentis* is vital given the significance of lentil production to the Canadian pulse industry and the current status of *C. lentis* as the economically most important lentil pathogen. Knowledge of mating type genes and the genetic pathways that regulate mating would help in assessing the potential for sexual recombination in the field.

CHAPTER 5

GENERAL DISCUSSION

The ascomycete fungus *Colletotrichum lentis* is the causal agent of anthracnose in lentil (Damm et al. 2014). *Colletotrichum lentis* has developed into a major fungal pathogen of lentil in Western Canada causing significant yield losses that have had negative effects on the Canadian pulse industry (Vail and Vandenberg, 2011). Although reported elsewhere in the world where lentil is grown, the origin of anthracnose in Western Canada remains unclear, with theories having being brought forward suggesting that *C. lentis* is an indigenous pathogen of native legume species in Western Canada and jumped hosts, or that this is another example of anthropogenic introduction (Morrall, 1997). Research so far indicates that the *C. lentis* population in Western Canada is made up of two pathogenic races (0 and 1), with sources of resistance to race 1 readily found in cultivated lentil, but higher levels of resistance to race 0 found only in wild lentil species (Buchwaldt et al. 2004; Shaikh et al. 2013). Recent research into the mating system of *C. lentis* revealed that the teleomorph can be induced *in vitro* (Armstrong-Cho and Banniza, 2006), and that *C. lentis* isolates can be grouped into two sexually incompatible groups (IG-1 and IG-2) as would be expected for typical ascomycete heterothallism. However, the genetic regulation of mating in *C. lentis* seems to deviate from the traditional heterothallic ascomycete mating as was already reported for other *Colletotrichum* spp. (Menat et al. 2012; Wheeler, 1954; Cisar and TeBeest, 1999; Vaillancourt et al. 2000). The purpose of this study was to examine the phylogenetic relationship of race 0 and race 1 isolates of *C. lentis* utilizing six loci (ACT, β-TUB, CHS-

1, GAPDH, HIS3 and ITS), and to gain a better understanding of the genetic regulation of the mating process in *C. lentis* by examining the mating type gene *MAT1-2*.

Phylogenetic analysis of the nucleotide sequence data from the six loci individually or combined, resulted in grouping of all 19 isolates of *C. lentis* in one unique cluster on the phylogenetic tree, displaying 100% sequence identity. This indicates an absence of genetic variability among the *C. lentis* isolates for the loci examined, confirming that the *C. lentis* pathogen population from Western Canada represents a single taxon. Therefore, clonal reproduction rather than different species explain the apparent linkage between incompatibility and race in field isolates of *C. lentis*, indicating that isolates of IG-2 in the founder population were exclusively race 0. It can also be speculated that race 0 isolates of the founder population originated in an environment where *L. ervoides* was present, considering that good resistance to it is present in this, but not the cultivated lentil species. As shown previously (Armstrong-Cho and Banniza, 2006; Gossen et al. 2009), conidial morphology and host range tests showed that *C. lentis* isolates were morphologically indistinguishable from each other and possessed the same host range with slight differences in disease severity on other leguminous and brassicaceous plant species tested.

In terms of the systematics, the phylogenetic position of *C. lentis* shows it to be part of the *destructivum* clade along with *C. linicola*, *C. higginsianum* and *C. destructivum*, in agreement with the recent comprehensive description of the *destructivum* clade (Damm et al. 2014). However, the isolate from *Matricaria perforata* currently designated as *C. truncatum*, is placed independently among the other members of the *destructivum*

species complex, and separate from the epitype specimen of *C. truncatum*. It has been demonstrated in the past, and shown here again, that *C. truncatum* ex *Matricaria perforata* can be easily mistaken for *C. lentis*, if morphology is the only tool employed (Forseille et al. 2011). The phylogenetic analysis certainly raises doubts over the taxonomy of isolates from *Matricaria perforata*, but sequence data from more isolates should be analyzed in order to clarify its taxonomy and phylogenetic relationship with *C. lentis* and the epitype specimen of *C. truncatum*, since *C. truncatum* isolates from *Matricaria perforata* are being considered for use as biocontrol agents to control *Matricaria perforata* in Western Canada (Peng et al. 2005). Multi-locus phylogeny in the current study also questions the taxonomic status of the anthracnose pathogen of *G. max*, currently designated as *C. truncatum*. All three isolates from *G. max* examined here were part of the *dematium* rather than *truncatum* clade including the epitype specimen of *C. dematium* which was supported by strong bootstrap values (99%) and posterior probability (1). Host range tests also showed that the *C. dematium* epitype infects *G. max* and causes similar disease severity to those induced by the isolates from *G.max*. Conidial morphology of *C. truncatum* and *C. dematium* is similar with both species having curved conidia and conidial dimensions of 21.8 x 1.9 µm for *C. truncatum* and 21.3 x 1.5 µm for *C. dematium* (Damm et al. 2009), which could lead to confusion in distinguishing between the two species.

Knowledge of host range helps in determining whether a pathogen is host specific and has limited distribution or possesses a wider host range and is probably opportunistic in nature. Host range testing conducted under controlled conditions on different

leguminous and brassicaceous cultivars proved that isolates of all species tested here were able to infect more than just their original host plant species. Notably, members of the *destructivum* clade possessed a highly similar host range spectrum, with *C. linicola* and *C. higginsianum* shown to be pathogenic on *L. culinaris* for the first time. The potential for any negative economic impact of *C. linicola* and *C. higginsianum* on *L. culinaris* and *Pisum sativum* needs to be evaluated further by examining a larger number of isolates *in vitro* as well as under field conditions, and by determining their prevalence in the environment. This study also demonstrated that both races of *C. lentis* can cause very high disease levels on *Pisum sativum* (90-93%) under controlled environmental conditions, whereas observations by Anderson et. al (2000) based on two field trials suggested *C. lentis* to only be weakly pathogenic on *Pisum sativum*. Interestingly, a new *Colletotrichum* species from *Pisum sativum*, *C. pisicola* shown to be part of the *C. destructivum* species complex was recently described by Damm et al. (2014), in addition to the previously known *C. pisi* reported from *Pisum sativum* in USA, Canada, Brazil, China, USSR and India (Farr and Rossman, 2014). Damm et al. (2014) also reported that the isolate (CBS 724.97) used to designate the new *C. pisicola* species possessed curved conidia uncharacteristic of members of the *destructivum* species complex and had been treated as *C. truncatum* in previous studies (Sheriff et al. 1994; Latunde-Dada and Lucas, 2007).

The phylum Ascomycota includes species that despite being distinct from each other on the basis of phylogeny and host range, exhibit interspecific fertility (Kaiser et al. 1997). Attempts to assess interspecific fertility in the current study showed that while *C. lentis*

isolates produced perithecia in intraspecific crosses, no perithecia were observed when *C. lentis* was mated with other closely related *Colletotrichum* species, irrespective of the plant material used. The number of perithecia in *C. lentis* varied with more being produced on lentil stems compared to non-host stems. Absence of sexual structures in crosses of *C. destructivum*, *C. truncatum ex P. lunatus* and *C. truncatum ex G. max* isolates on their host stems indicates that more crosses need to be performed in order to conclusively determine whether these species are homothallic or heterothallic or have lost fertility completely since notable differences in perithecia production have been reported in *Colletotrichum* spp. due to variation in fertility (Vaillancourt et al. 2000; Menat et al. 2012). It has been reported that numerous ascomycete fungi begin their sexual cycle only under specific conditions which could potentially serve as a reproductive barrier to mating with other individuals of other species (Poggeler et al. 2006). For instance, it was demonstrated through mating propensity assays that *Saccharomyces cerevisiae* did not hybridize with *S. paradoxis* as *S. cerevisiae* possessed a higher tendency of mating, leading to mating with other *S. cerevisiae* isolates instead of *S. paradoxis* which displayed a lower tendency of mating (Murphy et al. 2006). In *Neurospora*, circadian rhythms are known to control the expression of pheromone precursor genes, and variations in pheromone production were thought to be the reasons for lack of interfertility among *Neurospora* spp. (Bobrowicz et al. 2002; Karlsson et al. 2008).

All recent attempts to elucidate the mating system of species in the genus *Colletotrichum* have led to the conclusion that the process of sexual reproduction in

Colletotrichum spp. seems to be different from the typical ascomycete bipolar mating system. These inferences were made from *in vitro* mating experiments and MAT locus characterization studies that demonstrated discrepancies, such as the (1) presence of multiple alleles at a single MAT locus, (2) occurrence of two unlinked loci regulating cross fertility and, (3) presence of only the HMG domain of the *MAT1-2* in sexually compatible individuals and no detection of the *MAT1-1* α-box domain (Vaillancourt et al. 2000; Rodriguez et al. 2005, Garcia-Serrano et al. 2008; Menat et al. 2012). The model of 'unbalanced heterothallism' had previously been proposed stating that mutations in genes governing mating give rise to heterothallic behaviour in individuals that are otherwise homothallic in nature, resulting in sexual reproduction between individuals carrying complementary mutations (Wheeler, 1954).

In *C. lentis*, two sexually incompatible groups, IG-1and IG-2, are present with isolates of opposite IGs showing cross-fertility and resulting in the successful induction of perithecia despite the fact that all isolates contain only the HMG domain of the MAT1-2 idiomorph (Menat et al. 2012). Thus, the heterothallic phenotype seems to contradict the genotype, which could be considered to support the model of unbalanced heterothallism. The *MAT1-2* characterization in this study indicated that the *MAT1-2* genes of both IG-1 and IG-2 isolates do not carry any mutations, nor was any evidence of splice variants found, which could have led to an altered protein product varying in function. These results are consistent with research in *C. graminicola*, where the *MAT1-2* nucleotide sequences were identical in both mating partners (Vaillancourt et al. 2000) and also in *C. lindemuthianum* for which the *MAT1-2* gene in both parental isolates was

near identical (99%) with only a single base pair difference (Garcia-Serrano et al. 2008). A genetic study investigating cross fertility in *C. graminicola* revealed similar results to the mating studies in *C. lentis*. All *C. graminicola* isolates examined were self sterile and able to successfully produce ascospores by cross fertilizing with one of the parental isolates, but no isolate was able to mate with both parents. The HMG domain of *MAT1-2* was found in both mating partners. A sib crossing experiment led to the conclusion that two unlinked loci, *Crf1* and *Crf2*, were responsible for cross fertility in *C. graminicola* (Vaillancourt et al. 2000). Sequence identity for *MAT1-2* indicates that gene(s) other that *MAT1-2* are probably responsible for regulating sexual reproduction in *C. lentis*, and other species of this genus.

Mating type (*MAT*) genes encode transcriptional factors that regulate the expression of genes necessary for sexual reproduction. *MAT* gene functions in the self- incompatible ascomycete *N. crassa* have been studied in detail through gene mutations and it has been demonstrated that *MAT1-1* and *MAT1-2* are critical not only for fertilization, but also for events such as internuclear recognition, formation of the ascogenous hyphae and fruiting body development that follow fertilization (reviewed by Debuchy and Turgeon, 2006). MAT locus deletion resulted in complete loss of mating ability in *N. crassa* (Ferreira et al. 1998). The entire α1 domain and subsequent 277 residues of the *MAT1-1* and the HMG domain with its C-terminal region of the *MAT1-2* are absolutely essential for mating in *N. crassa* (Philley and Staben, 1994). A search for downstream targets of the mating type DNA binding transcription factors α1 domain (*MAT1-1*) and HMG domain (*MAT1-2*) led to the identification of genes coding for pheromone

precursors and pheromone receptors as putative targets. For example in *N*. *crassa* the *pre-1* and *pre-2* pheromone receptor genes were identified in MAT1-1 and MAT1-2 isolates, respectively (Kim and Borkovich, 2004). It has also been demonstrated that the MAT proteins regulate genes that are involved in post-transcriptional modifications of pheromone precursors (Coppin et al. 2005).

A genome-wide gene expression study of the vegetative mycelium from individuals of opposite mating type in *P. anserina* revealed that the mating type transcriptional factors directly or indirectly regulate a much larger number of target genes than those in yeasts. Also, many of these putative target genes have no direct role in mating but seem to control biological processes like metabolism and energy production. Only pheromone and pheromone receptor genes targeted by mating type genes were considered indispensable for mating to occur in *P. anserina*. It was hypothesized that the development of perithecia is dependent on nutrition provided by the mycelium and the mating type genes could be responsible for transport of these nutrients to the growing perithecium via regulation of target genes that are not involved in the mating process directly (Bidard et al. 2011). In the gene expression study undertaken here, *MAT1-2* was shown to be constitutively expressed in the vegetative mycelia from both the parental isolates CT-21 and CT-30 of *C. lentis*, irrespective of whether they were cultured separately or co-cultured. This suggests that *MAT1-2* could be a regulator of non-mating target genes, as seen in the case *of P.* anserina, providing the essential nutrients for perithecium development in *C. lentis*.

To conclude, this study confirmed that *C. lentis* isolates from the two pathogenic races belong to a single taxon, clearly evident from the multi-locus phylogenetic analysis, and also supported by conidial morphology, mating studies and host range approaches. Sequence identity and constitutive expression of the mating type gene *MAT1-2* between sexually compatible isolates indicated that *MAT1-2* is not responsible for regulating the mating process in *C. lentis*.

In order to determine whether *C. lentis* is in fact an indigenous pathogen, future studies should explore the host range of *C. lentis* further by inoculating isolates of race 0 and 1 onto leguminous plant species that are native to the Canadian prairies. *Colletotrichum lentis* has been reported from other countries so mating studies between *C. lentis* isolates from Western Canada and isolates from other countries should be undertaken to determine if outcrossing also takes place among these isolates. Also, studies focusing on determining the functions of *MAT1-2* and its role in the mating process of *C. lentis* should be carried out by performing gene knock-outs or knock-down experiments. The availability of the genome sequence of *C. lentis* will help in discovering the *MAT1-2* target genes but also in identifying candidate genes that may be involved in regulating the mating process in *C. lentis*, through strategies such as mutational analysis and genome-wide transcriptome profiling.

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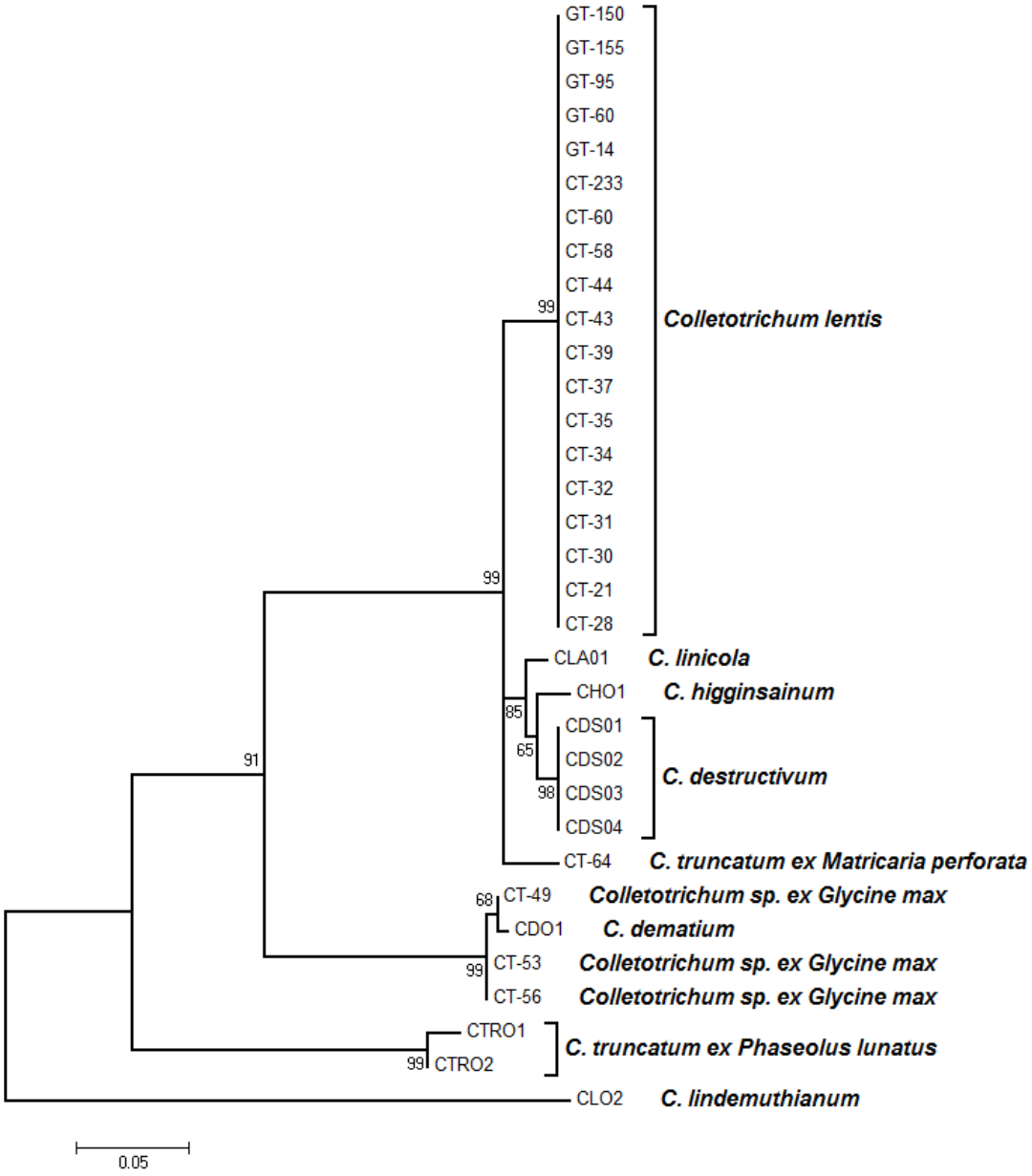
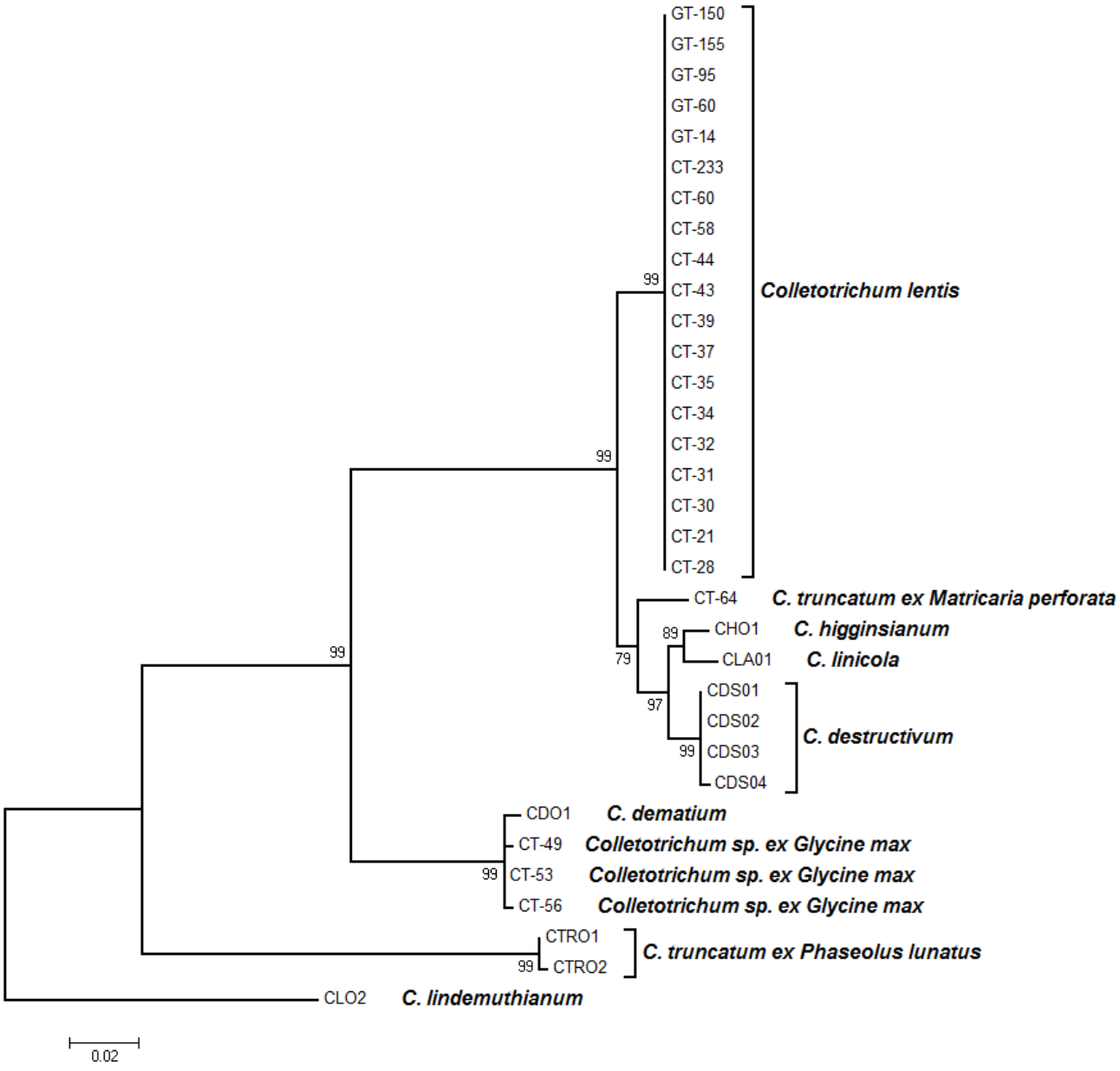
APPENDICES

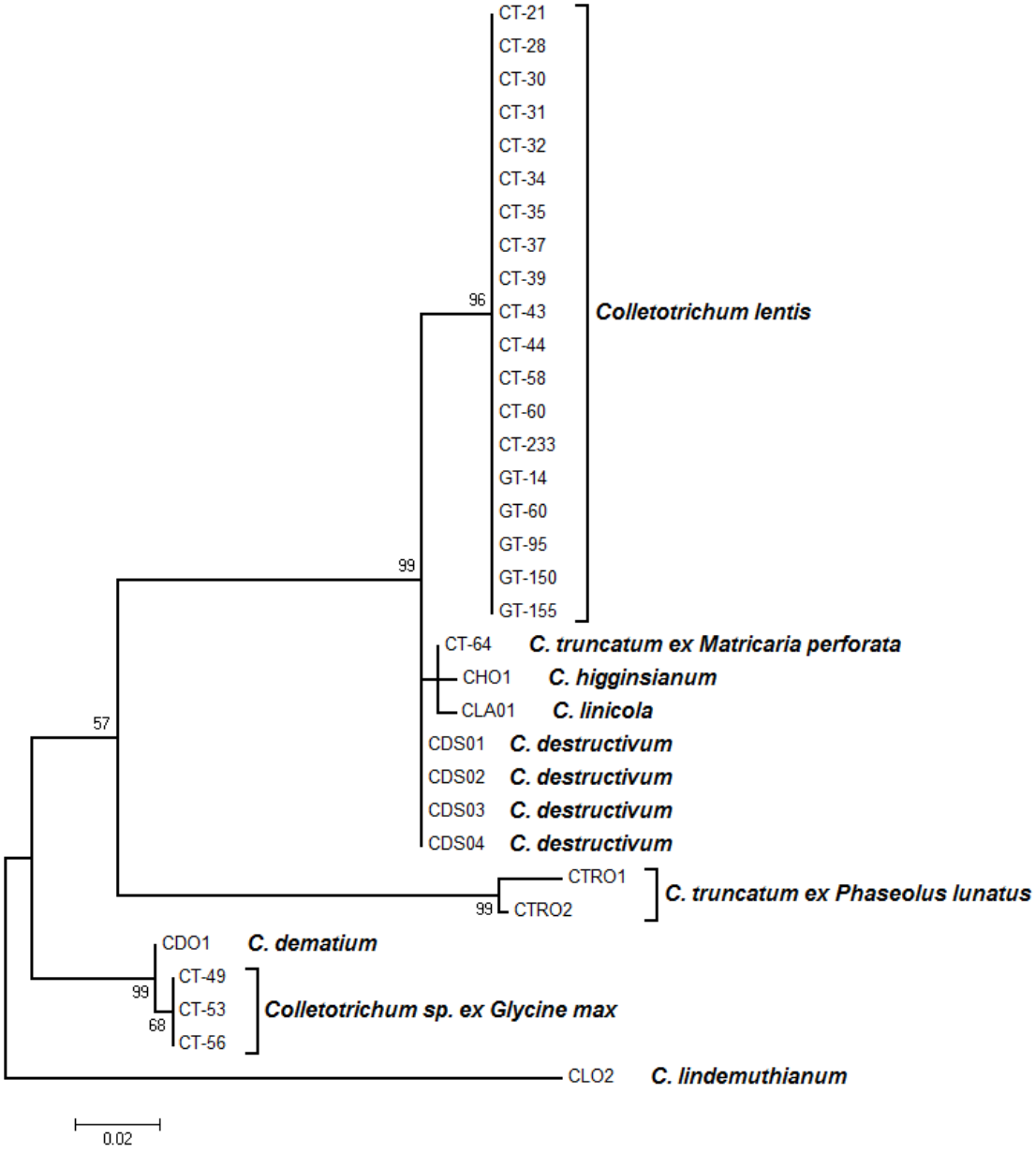
APPENDIX 1. Phylogenetic tree derived from *ACT* sequences of *Colletotrichum* spp. using Maximum likelihood analysis. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap analysis (1000 replicates) is shown next to the branches.



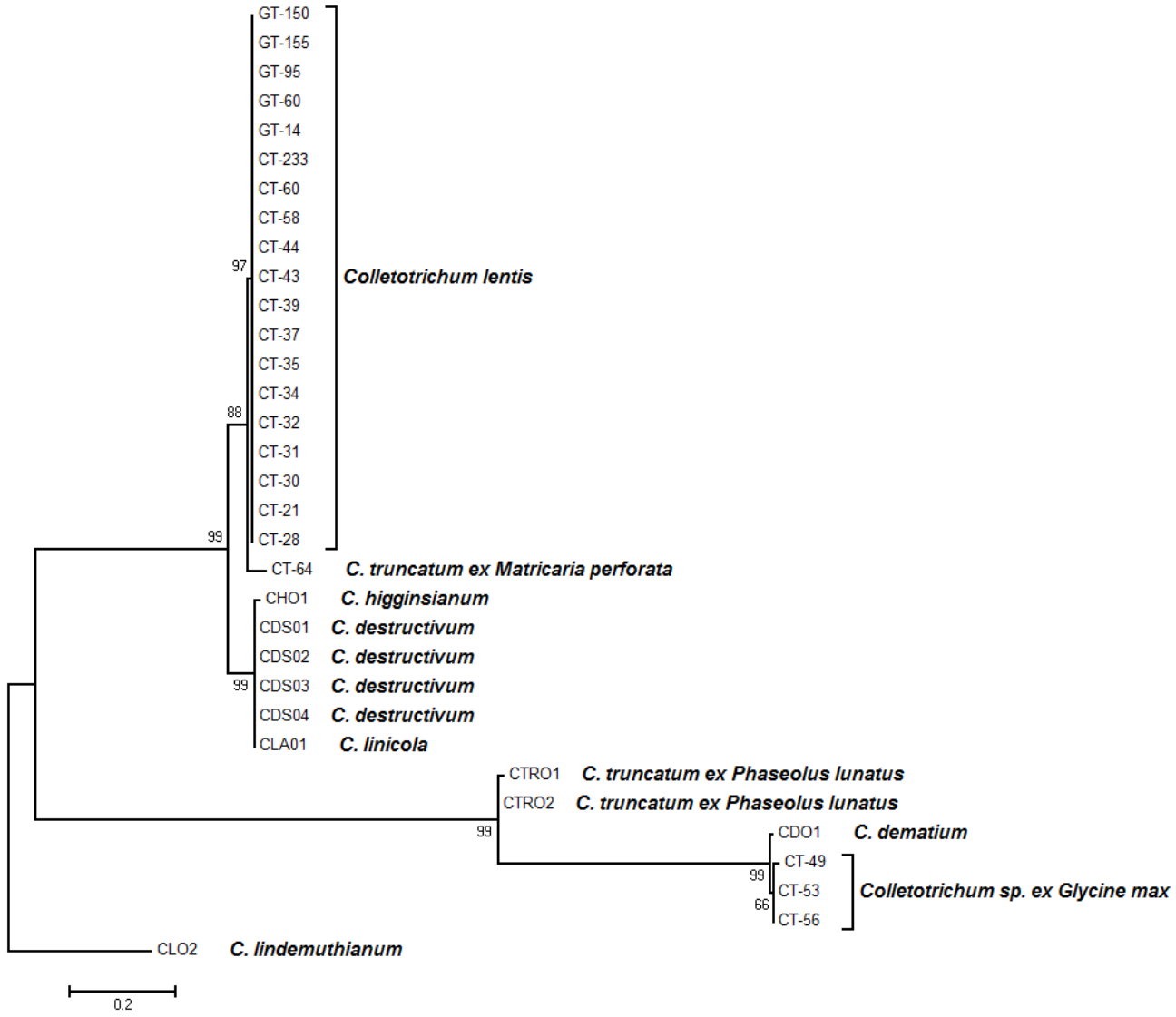
APPENDIX 2. Phylogenetic tree derived from *β-TUB* sequences of *Colletotrichum* spp. using Maximum likelihood analysis. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap analysis (1000 replicates) is shown next to the branches.



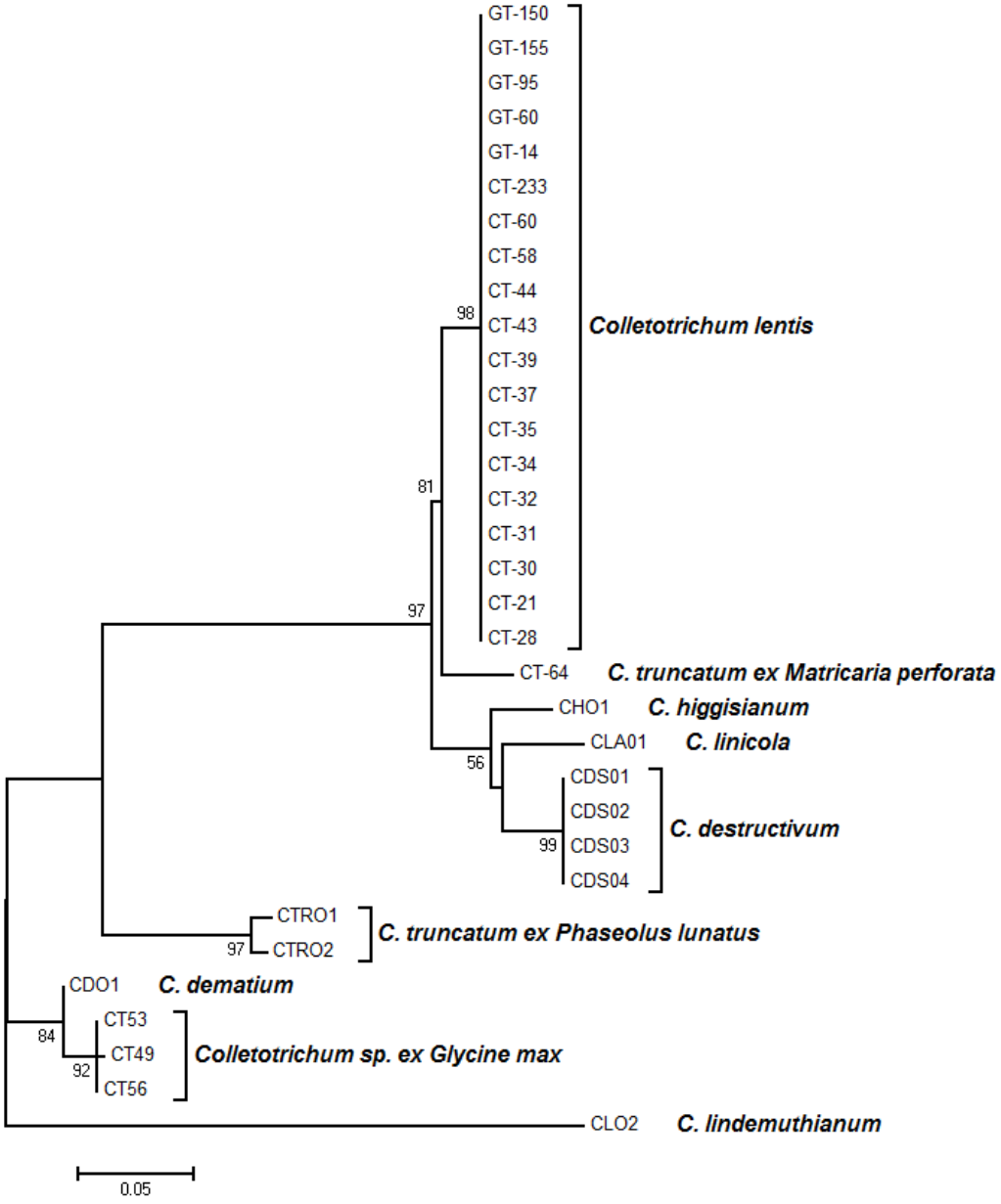
APPENDIX 3. Phylogenetic tree derived from *CHS-1* sequences of *Colletotrichum* spp. using Maximum likelihood analysis. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap analysis (1000 replicates) is shown next to the branches.



APPENDIX 4. Phylogenetic tree derived from *GAPDH* sequences of *Colletotrichum* spp. using Maximum likelihood analysis. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap analysis (1000 replicates) is shown next to the branches.



APPENDIX 5. Phylogenetic tree derived from *HIS3* sequences of *Colletotrichum* spp. using Maximum likelihood analysis. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap analysis (1000 replicates) is shown next to the branches.



APPENDIX 6. Phylogenetic tree derived from *ITS* sequences of *Colletotrichum* spp. using Maximum likelihood analysis. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap analysis (1000 replicates) is shown next to the branches.